MATERNAL RECOGNITION OF PREGNANCY IN THE EWE: THE STRUCTURE/FUNCTION RELATIONSHIP OF OVINE INTERFERON TAU

By

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TABLE OF CONTENTS

LIST OF FIGURES

KEY TO ABBREVIATIONS

aa ... Amino Acid

Ala ... Alanine

ANOVA... Analysis of Variance

Arg ... Arginine

AVP ... Arginine Vasopressin

CT ... Carboxyl-terminus of oIFNτ; aa 139-172

Cys ... Cysteine

DNA ... Deoxyribonucleic Acid

GLM ... General Linear Models

Gln ... Glutamine

Glu ... Glutamic Acid

HSP ... Heat Shock Protein

IFN ... Interferon

IFNr ... Type I Interferon Receptor

IFNα ... Interferon alpha

IFNß ... Interferon beta

IFNω ... Interferon omega

IRF-2 .. Interferon Regulatory Factor-2

IRF-E .. Interferon Regulatory Factor Element

ISGF3 .. IFN Stimulated Gene Factor-3 Complex

ISGF3α . IFN Stimulated Gene Factor-3α

ISGF3γ . IFN Stimulated Gene Factor-3γ

ISRE ... IFN Stimulated Response Element

IP ... Inositol Phosphate

JAK ... Janus Kinase

KRB ... Kreb's Ringer Bicarbonate

Leu ... Leucine

mRNA ... Messenger Ribonucleic Acid

NT ... Amino-terminus of oIFNτ; aa 1-37

oCSP ... Ovine Conceptus Secretory Protein

oIFNτ .. Ovine Trophoblast Interferon

OT ... Oxytocin

oTP-1 .. Ovine Trophoblast Protein-1

OTr ... Oxytocin Receptor

PEG ... Polyethyleneglycol

Pep 2... aa 34-64 of oIFNτ

Pep 3... aa 62-92 of oIFNτ

Pep 4... aa 90-122 of oIFNτ

Pep 5... aa 119-150 of oIFNτ

$PGF_{2\alpha}$ .. Prostaglandin-$F_{2\alpha}$

PGFM ... 13,14-dihydro-15-keto Prostaglandin-$F_{2\alpha}$

Pro ... Proline

RNA ... Ribonucleic Acid

rbIFN . Recombinant Bovine Interferon-alpha

roIFNτ . Recombinant Ovine Trophoblast Interferon

SAS ... Statistical Analysis System

SEM ... Standard Error of the Mean

Ser ... Serine

Stat1α.. Signal Transducers and Activators of Transcription-1α

Stat1ß.. Signal Transducers and Activators of Transcription-1ß

Stat2 .. Signal Transducers and Activators of Transcription-2

SP ... Serum Protein

Tyk2 ... Tyrosine Kinase

Tyr ... Tyrosine

Trp ... Tryptophan

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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In the ewe, pregnancy success is dependent on the conceptus secreting the pregnancy recognition factor, oIFNτ, during the maternal recognition of pregnancy period. oIFNτ is secreted by the trophoblast cells of the developing conceptus. Binding to specific endometrial receptors initiates a series of events which block the release of luteolytic $PGF_{2\alpha}$. Our current working hypothesis is that oIFNτ accomplishes this attenuation of pulsatile $PGF_{2\alpha}$ secretion by the endometrium, through preventing expression of the Er gene, and subsequent up-regulation of expression of the OTr gene that normally occurs in non-pregnant ewes. The NT portion of the oIFNτ has been shown, through competitive binding studies, to act through a specific domain of the Type I IFN receptor while the CT is believed

to act through a more common domain of that receptor. The present studies were conducted to determine if the NT, or the other portions of oIFNτ, has pregnancy recognition properties when injected into the uterine lumen of cyclic ewes. Intrauterine injections were either one of six overlapping synthetic oIFNτ peptides (NT was the peptide most closely examined), ovine conceptus protein or roIFNτ. The NT peptide was nearly as effective as roIFNτ in suppressing expression of endometrial Er and OTr, blocking the metabolism of inositol phosphate in endometrial tissues after an oxytocin challenge test *in vitro* and PGFM response to an oxytocin challenge test *in vivo*. NT had no effect on endometrial Pr expression. Collectively, these results support the hypothesis that oIFNτ suppresses expression of endometrial Er and OTr, thereby preventing the pulsatile release of $PGF_{2\alpha}$ without affecting expression of Pr. Also, these results indicate that the NT peptide is as effective as oIFNτ in producing a pregnancy recognition response. This finding supports the hypothesis that the NT of oIFNτ is the most likely portion of oIFNτ responsible for the antiluteolytic properties of oIFNτ which distinguish it from other Type I IFNs.

CHAPTER 1
GENERAL INTRODUCTION

The "term maternal recognition of pregnancy" was first used in 1969 by R.V. Short. Identification of the maternal pregnancy recognition factor as an IFN came about as the result of molecular cloning and amino acid sequencing (Stewart *et al.*, 1987; Imakawa *et al.*, 1987, 1989; Charpigny *et al.*, 1988). These reports identified the maternal pregnancy recognition factor, referred to currently as oIFNτ, as a Type I IFN. Trophoblast IFNs are biologically similar to other Type I IFNs, and like the other IFNτs, oIFNτ displays both antiviral and antiproliferative properties as does IFNα (Pontzer *et al.*, 1988). Ovine IFNτ binds to high affinity (Godkin *et al.*, 1984a) Type I IFN receptors (Stewert *et al.*, 1987) which are distributed throughout endometrial tissues of the ewe and their expression may be influenced by ovarian steroids (Knickerbocker and Niswender, 1989). Synthetic peptides corresponding to the amino- (Pontzer *et al.*, 1991) and carboxyl-terminal peptides of oIFNτ, as well as two internal peptides (aa 62-92 and aa 119-150) were found to inhibit oIFNτ receptor binding and antiviral activity in a dose

dependent manner (Pontzer *et al.*, 1994) indicating specific competition between these peptides and oIFNτ. The NT had no effect on the antiviral activity of IFN. These findings indicated that the NT may act through a novel domain of the oIFNτ receptor while the other peptides may act through a more common domain of the Type I IFN receptor. This could explain the unique actions of oIFNτ.

Ovine IFNτ-induced hormone action is initiated by the transduction of signal via activation of four cytosolic proteins which bind to interferon stimulated response elements (see Williams, 1991a; Darnell *et al.*, 1994) and is believed to act in the same manner as all other Type I interferons. Ovine IFNτ and other Type I IFNs increase endometrial protein production dramatically (Gross *et al.*, 1988b; Sharif *et al.*, 1989; Ashworth and Bazer, 1989; see Spencer *et al.*, 1996) and affect expression of Er concentration within the endometrium during early pregnancy (Mirando *et al.*, 1993; Ott *et al.*, 1993b; Wathes and Hamon, 1993; Spencer *et al.*, 1995a, 1995b) to allow for establishment of pregnancy.

In the cyclic ewe, the Er is abundant just before, during and after estrus. Receptor numbers fall dramatically from Day 3, to a low on Days 10 to 14, followed by a dramatic increase beginning on Day 14, to peak at estrus (Koligian and Stormshak, 1976; Miller *et al.*, 1977; Zelinski *et al.*, 1980; Cherny *et al.*, 1991; Ott *et al.*, 1993b;

Spencer *et al.*, 1996). Progesterone is inhibitory to Er formation in most species (Brenner *et al.*, 1974; Hsueh *et al.*, 1975 and 1976; Tseng and Gurpide, 1975; West *et al.*, 1976; Bhakoo and Katzenellenbogen, 1977) including sheep (Koligian and Stormshak, 1977b; Zelinski *et al.*, 1980; Cherny *et al.*, 1991). Progesterone in the cyclic animal is clearly inhibitory to its own endometrial receptor, probably through a down-regulation mechanism (Milgrom *et al.*, 1973; Leavitt *et al.*, 1974; Vu Hai *et al.*, 1977; Spencer *et al.*, 1996). Progesterone's down-regulation of its own receptor initiates removal of the proposed progesterone block (McCracken *et al.*, 1984), allowing Er up-regulation, and formation of OTr which bind oxytocin to initiate pulsatile release of $PGF_{2\alpha}$ from the uterus.

In the cyclic ewe luteolysis is initiated by pulsatile release of $PGF_{2\alpha}$ produced by the uterus (McCracken *et al.*, 1981; Hooper *et al.*, 1986; Thornburn *et al.*, 1973; Niswender and Nett, 1994). Oxytocin from the ovary, binding to its endometrial OTr, and $PGF_{2\alpha}$ from the uterus binding to its ovarian receptor, act together in a positive feedback loop to generate the pulses of $PGF_{2\alpha}$ required for luteolysis (Flint and Sheldrick, 1983; Hooper *et al.*, 1987; Niswender and Nett; 1994). Endometrial OTr begin to increase on Day 14, reach maximum values at estrus and decline by Day 5 of the subsequent cycle. This is coincidental with the decrease in plasma progesterone and the rise in estrogen

(Sheldrick and Flint, 1985; Wallace *et al.*, 1991) during proestrus.

Intrauterine injections of conceptus homogenates increase the interestrous interval in ewes (Rowson and Moor, 1967) and oCSPs elicit the same response (Godkin *et al.*, 1984b; Vallet *et al.*, 1988). Intrauterine injection of oCSPs also blocks cyclic uterine $PGF_{2\alpha}$ responsiveness after oxytocin challenge (Fincher *et al.*, 1986; Vallet *et al.*, 1988; Mirando *et al.*, 1990a). The lack of $PGF_{2\alpha}$ responsiveness (Fincher *et al.*, 1986; Vallet *et al.*, 1988) and IP hydrolysis (Mirando *et al.*, 1990b) in the pregnant ewe is due to low OTr (Flint and Sheldrick, 1986) and Er (Findlay *et al.*, 1982) on the luminal epithelium (Spencer *et al.*, 1996). The proposed progesterone block (McCracken *et al.*, 1984) to OTr formation was initially believed to be conceptus maintained by preventing the normal down-regulation of the Pr on Days 12 to 14. Recent results of Spencer *et al.* (1995a,b) indicate that in the pregnant ewe oIFNτ prevents luteolysis, not by stabilizing Pr and maintaining the progesterone block, but by preventing expression of the Er gene which, in turn, prevents expression of the endometrial OTr gene.

Present studies determined the function of oIFNτ on factors associated with maternal recognition and examined the functional properties of specific domains of oIFNτ responsible for maternal recognition of pregnancy effects.

The experimental designs and methods for the five individual animal experiments which comprise the present studies are discussed in Chapter 3 and are referred to throughout the remainder of this dissertation by experiment number.

CHAPTER 2
REVIEW OF THE LITERATURE

Arachidonic Acid Metabolism And Prostaglandin Synthesis

Arachidonic acid, a 20 carbon fatty acid, is supplied through the diet or by anabolic metabolism of linoleic acid (Ramwell, 1977). Most arachidonic acid is present within integral membrane components of cells in the form of phospholipids. Arachidic acid is found most prominently at the 2n position of phosphatidylcholine, phosphatidylethanolamine, and, to a lesser extent, phosphatidylinositol (MacDonald and Sprecher, 1991). Arachidonic acid, once mobilized from phospholipids, usually enters into one of three major arachidonic acid metabolic cascades; the cyclooxygenase, lipoxygenase, or epoxygenase pathways. The cyclooxygenase pathway produces prostaglandins and thromboxanes (Smith *et al.*, 1991). The lipoxygenase pathway produces leukotrienes, hydroxy acids and lipoxens (Samuelsson, 1987; Smith *et al.*, 1991). Epoxy acids and dihydroxy acids are formed via the epoxygenase pathway (Fitzpatrick and Murphy, 1988).

Arachidonic acid is mobilized from phospholipids in response to extracellular stimuli, in part, through the

actions of one of three phospholipases; phospholipase A_1, A_2, and C (Smith, 1986). Phospholipase A_1 cleaves acyl residues from phospholipids at the 3n (3 carbon) position of the glycerol backbone to produce a lysophospholipid from which arachidonic acid is then cleaved. Phospholipase A_2 mobilizes arachidonic acid directly from the phospholipid at the 2n position of the glycerol backbone (Loeb and Gross, 1986; Kramer *et al.*, 1989;). Phospholipase C, via receptor-mediated G protein stimulation, cleaves phosphatidylinositol into inositol 1,4,5-trisphosphate and diacylglycerol, each of which can act as a transduction signal within the cell. Arachidonic acid is subsequently cleaved from diacylglycerol at the 2n position by an inositol specific phospholipase C enzyme with both diacylglycerol lipase and monoacylglycerol lipase activities (see Berridge, 1984; Smith *et al.*, 1991). Arachidonic acid mobilization for prostaglandin synthesis is primarily through the actions of phospholipase A_2 and phospholipase C (Martin and Wysolmerski, 1987).

The prostaglandins -A, -D, -E, -F, and -I, are 20 carbon compounds produced via the cyclooxygenase pathway of arachidonic acid metabolism, through the actions of prostaglandin-H synthetase (Smith, 1989). Prostaglandin-H synthetase is an integral membrane protein found on the cytoplasmic side of cell membranes (Smith *et al.*, 1991). Prostaglandin-H synthetase exhibits both cyclooxygenase and hydroperoxidase activities as separate sites on the

synthetase enzyme (Miyamoto *et al.*, 1976; Pagels *et al.*, 1983) Cyclooxygenase catalyzes prostaglandin-G_2 formation from arachidonic acid and hydroperoxidase acts on the 15-hydroperoxyl group of prostaglandin-G_2 to form prostaglandin-H_2. Both cyclooxygenase and hydroperoxidase activities require heme (Kartheim *et al.*, 1987). Prostaglandin production is strongly regulated by the availability of substrate (arachidonic acid) and control of prostaglandin-H synthetase activity to produce prostaglandin-H_2, the precursor of all prostaglandins and thromboxane.

Prostaglandin-D_2, prostaglandin-E_2, prostaglandin-I_2, and thromboxane are produced from prostaglandin-H by non-oxidative rearrangements through the action of their respective synthetase enzymes. Prostaglandin-$F_{2\alpha}$ can be formed by three separate mechanisms through prostaglandin-F synthetase enzyme actions. Prostaglandin-$F_{2\alpha}$ can be formed from prostaglandin-H_2 through endoperoxide reductase activity or reduction from prostaglandin-E_2 by a 9-keto-reductase. An active metabolite of $PGF_{2\alpha}$, 9α,11ß-$PGF_{2\alpha}$ can be produced from prostaglandin-D_2 via 11-keto-reductase (Smith *et al.*, 1991).

Interferon Receptors/Signal transduction

Interferons can be divided into two general types (for review, see Roberts *et al.*, 1992). Type II interferon is

the product of a single gene and is known as IFNγ. Type I IFN is composed of at least three distinct subtypes known as IFNα, IFNß and IFNω. Although these three subtypes differ markedly in amino acid sequence, they elicit their actions through a common receptor and exhibit similar biological properties. Although commonly thought to be derived primarily from T-lymphocytes and natural killer cells in the presence of other cells infected with virus, Type II IFN has been shown to be released from a variety of cells, including porcine trophoblast (Lefevre *et al.*, 1990). Type-I IFNs can be induced by many cells within the body. Interferon-α, produced by immune cells, is the most prominent, while IFNτ is produced exclusively by trophoblast cells. It is IFNτ, a member of the Type-I IFN family which is involved in pregnancy recognition, therefore, this discussion on IFN receptors and signal transduction will be limited to the Type-I IFN family.

The Type-I IFN receptor is a transmembrane receptor comprised of two subunits (α and ß; Platanias *et al.*, 1994) Coupling of IFN to its receptor sets into motion a series of phosphorylations of proteins within the cell which ultimately affect transcription of various proteins. How specificity is acquired is not understood at this time as apparently all Type-I IFNs bind to the same or similar receptors and initiate their responses through the same transduction signaling pathway (see, Johnson *et al.*, 1994).

Signal transduction for IFNs is not through stimulation of CAMP, cGMP, or IP turnover (see Bazer, 1992; Bazer *et al.*, 1993; Johnson *et al.*, 1994). Rather, IFNs elicit their action on gene function through a complex signal transduction cascade involving activation of protein tyrosine kinases including those belonging to the Janus kinase (Jak) family, including Tyk2 and Jak1 (for review, see Darnell *et al.*, 1994). Ligand binding initiates the activation of Tyk2, which is associated with the cytoplasmic domain of the Type-I IFN receptor. Either ligand binding or activation of Tyk2 results in activation of Jak1. These two kinases, either separately or together, act to phosphorylate proteins within the cytoplasm which comprise the interferon stimulated gene factor-3 complex (see, Pfeiffer *et al.*, 1994, Johnson *et al.*, 1994). These subsequently translocate to the nucleus where they bind to specific interferon stimulated response elements on DNA sequences that direct IFN-induced transcriptional responses. interferon stimulated gene factor-3 is actually two complexes composed of four separate components which normally reside in a dissociated form in untreated cells. The interferon stimulated gene factor-3α complex forms within minutes following IFN treatment, and is composed of three proteins (113 kDa STAT2, 91 kDa STAT1α and 84 kDa STAT1ß; Fu *et al.*, 1992; David and Larner, 1992). It has been suggested that Jak1 is the kinase responsible for activating STAT1 while

Tyk2 phosphorylates STAT2 (Silvennoinen *et al.*, 1993; Darnell *et al.*, 1994). Inhibitors of the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism increase activity of interferon stimulated gene factor-3α (Hannigan *et al.*, 1991). This indicates that a factor produced from metabolism of arachidonic acid in the epoxygenase pathway may amplify the interferon stimulated gene factor-3α signal (Hannigan *et al.*, 1991; Williams, 1991b). Once activation and binding of these three components occurs, interferon stimulated gene factor-3α binds a fourth DNA-binding protein (48 kDa interferon stimulated gene factor-3γ; Levy *et al.*, 1990; see Marx, 1992), and the multimeric complex translocates to the nucleus and binds to the interferon stimulated response elements (Fu, 1992; Schindler *et al.*, 1992; Larner *et al.*, 1993).

Interferon-stimulated response elements are cis-acting DNA elements found upstream in IFN responsive genes which activate transcription when bound (see Williams, 1991b). Transcription is activated after binding of the activated interferon stimulated gene factor-3 complex, made up of interferon stimulated gene factor-3α and interferon stimulated gene factor-3γ, to interferon stimulated response elements.

The consensus sequence of interferon stimulated response elements for Type I IFNs is reported to have both a

common motif (GGAAA) and a specific motif (TGAAACT). The specific motif is reportedly separated from the common motif by 1 residue (3') (see Williams, 1991a; Kerr and Stark, 1991). Once the interferon stimulated response element is bound, RNA polymerase II initiates transcription.

Other members of the Type I IFN regulatory factor family also play a major role in IFN action. The interferon regulatory factor-1 gene contains an interferon stimulated response element, which when bound by the activated interferon stimulated gene factor-3 complex, is up-regulated, resulting in an increase in the positive transcription factor interferon regulatory factor-1. The interferon regulatory factor-1 protein, in contrast to the interferon stimulated gene factor-3 complex, can in turn bind to an interferon regulatory factor element, which is often contained within a larger interferon stimulated response element, and increase gene transcription. One such gene which contains a interferon regulatory factor element is the interferon regulatory factor-2 gene, and binding to this interferon regulatory factor element by interferon regulatory factor-1 results in an increase in the production of the transcription factor interferon regulatory factor-2. interferon regulatory factor-1 and interferon regulatory factor-2 are structurally similar DNA-binding factors which have contrasting roles in IFN action. interferon regulatory factor-1 serves as a transcriptional activator of IFN and

IFN-inducible genes, while interferon regulatory factor-2 represses interferon regulatory factor-1 action by competing for the same cis elements and displacing interferon regulatory factor-1 (Harada *et al.*, 1994). Thus, this "yin-yang interaction", as described by Spencer *et al.* (1996), modulates IFN action by regulating the induction and repression of Type I IFN-responsive gene expression.

Steroid Receptors

Models For Steroid Receptors; Historical Perspective

Mueller *et al.* (1958) was the first to propose a mechanism of action for a steroid. This report led to a search for the compartment in which the steroid receptor was located. Toft and Gorski (see Gorski *et al.*, 1968) used sucrose gradient centrifugation to separate the cytosolic and nuclear fractions of cells treated with tritiated estradiol. They found most of the radioactivity in the nuclear fraction. Only after an excess of estradiol was added was there detectable radioactivity in the cytosol. However, previous studies indicated the presence of unbound receptor in the cytosol. This led to studies which examined the effects of time and temperature, during incubation with estradiol, on location of the Er. In these studies whole uteri were incubated at 0°C and 37°C. After 1 h at 37°C there was more estrogen in the nuclear fraction than in the

cytosolic fraction. However, at 0°C most estradiol was in the cytosol. Incubation of uteri for 1 min at 0°C with labeled estradiol, followed by transfer of the tissues to fresh medium without estradiol and incubation at 37°C, resulted in the appearance of estradiol in the nuclear fraction. Because formation of the nuclear fragment (9.5s) resulted in loss of the estrogen-bound cytosolic fragment (4-6s) the question was whether the cytosolic fragment (4-6s) was extracellular in origin and whether estrogen from the 9.5s fraction was from the cytosol fragment. This was answered by experiments utilizing cell-free preparations of cytosol incubated with labeled estradiol. The cell-free cultures prepared from uteri incubated at 0°C with labeled estrogen, followed by incubation at 37°C without estrogen had very little estrogen in the cytosolic fraction. However, if the same experiment was conducted without estrogen during the 0°C incubation the estrogen was found in the cytosol. These results led the authors to propose that estrogen moved from the cytosol to the nucleus and, in the process, the 9.5s fragment was lost. Autoradiographic studies by Jensen *et al.* (1968) showed that uterine tissues incubated at 37°C with labeled estradiol had the majority of the label within the nucleus. However, when incubations were conducted at 2°C the majority of the label was located within the cytosol. These findings were supported by earlier immunocytochemical studies.

This led to, a revision of the proposal for the translocation model for the steroid receptors (Gorski *et al.*, 1968). In this model, estrogen diffuses into cells from the blood and binds to a cytoplasmic receptor. The binding of estrogen induces a conformational change in the receptor which allows movement of the ligand-receptor complex from the cytosol to the nucleus. This conformational rearrangement changes the shape of the receptor and causes the release of one or more proposed subunits attached to the receptor in the inactive state. It was this combination of the loss of the subunits and the conformational change that caused the proposed change from the 9.5s cytosolic form to the 4-6s nuclear form. Once in the nucleus, it was believed that receptor-initiated events resulted in increased cellular protein production by estrogen-treated uterine tissues.

Current Model For Steroid Receptors

In recent years, improved separation techniques, utilizing a cytochalasin enucleation procedure, allowed more complete separation of the cytosolic and nuclear fractions into cytoplasts and nucleoplasts, respectively. With this procedure, Welshons *et al.* (1984, 1985) conclusively established that Er, Pr and Gr are located within the nucleus. Techniques, using frozen tissue for immunocytochemical detection of steroid receptor by

monoclonal antibody binding, also indicated that the unoccupied receptor for estrogen was in the nucleus (King and Greene, 1984). In light of these findings, Gorksi *et al.* (1986) proposed the current model for steroid receptor action. This model is very similar to that previously described except that the unoccupied receptor is found only in the nuclear compartment. The steroid diffuses into the nucleus, binds to the specific receptor which then undergoes conformational changes, and the receptor-ligand complex binds to the steroid response element in the promotor/enhancer region of DNA.

Structure/Function Of The Steroid Receptor

Steroid receptors belong to a super-family of nuclear receptors that include receptors for all steroids, retinoic acid, thyroid hormone, vitamin D, and several other receptors which are termed orphan receptors. Orphan receptors are not fully characterized but are apparently involved in proper development in Drosophila. There is a high degree of homology among the steroid receptors, and all members of this super-family have common functional domains which account for their high homology. These domains consist of a DNA binding domain, a ligand binding domain, a dimerization domain, a heat shock protein 90 binding domain, a nuclear localization domain and a transactivation domain

(for reviews see Carson-Jurica *et al.*, 1990; Wahli and Martinez, 1991; Freedman, 1992; and Orit *et al.*, 1992).

The DNA binding domain, probably the most studied, is noted for many conserved cysteines. Eight of the cysteines are arranged into zinc finger complexes where four cysteines in each of the two fingers bind a zinc ion. The DNA binding domain of the steroid super-family is divided into two separate subfamilies based on differences in the zinc fingers which are involved in DNA sequence recognition and receptor dimerization. These subfamilies are the glucocorticoid/progesterone subfamily, which includes androgen receptors and mineralocorticoid receptors, and the estrogen subfamily, which includes retinoic acid, thyroid hormone, vitamin D, and the orphan receptors

The DNA binding domain of the glucocorticoid receptor was the first to be characterized. Subsequent studies indicated that only a portion of this domain was required for DNA binding and that the DNA binding domain also possessed hormone-dependent transcriptional activity (Hollenberg *et al.*, 1987). As stated previously, this domain has two zinc finger structures similar to those originally reported in transcription factor IIIA. Cloning and sequencing of the steroid receptor zinc finger region led to the following consensus sequence: Cys-X_2-Cys-X_{13}-Cys-X_2-Cys-X_{15}-Cys-X_5-Cys-X_2-Cys-X_4-Cys (see Freedman, 1992).

The ligand binding domain (220 - 250 amino acids) located near the carboxyl terminal includes domains for dimerization, transactivation and heat shock protein 90 binding. The N-terminal domain is highly variable in length and exhibits the conserved least sequence homology of all the domains. It is, therefore, sometimes referred to as the immunologic domain to which antibodies are generated to distinguish between the various receptors within this super-family. This domain also contains an area involved in transactivation.

Receptor Activation

Prior to ligand binding the steroid receptor is held in an inactive state by inhibitory proteins; heat shock protein 90 is the primary protein responsible for this inhibition. Salt treatment of steroid receptors removes heat shock protein 90 and causes activation of the receptor. The heat shock protein 90 binding occurs in the carboxyl terminal region of the receptor; the same area to which the ligand binding domain has been mapped. Apparently there are two molecules of heat shock protein attached to each receptor molecule. Studies which utilized fragments of the glucocorticoid receptor indicated that heat shock protein 90 may be involved in maintaining conformation of the receptor, such that the ligand is unable to bind. When the heat shock protein was removed, ligand binding was blocked (Bresnick *et al.*, 1989) possibly due to protein unfolding characteristics

reported for other heat shock proteins (Pelham, 1988). The two heat shock protein 90 molecules are lost as a result of a conformational changes induced by ligand binding. Conformational change causes movement of the hinge region, loss of the heat shock protein 90 molecules and exposure of the DNA binding domain to allow binding to hormone response elements. This also allows formation of a dimmer of two steroid receptor molecules which is necessary for binding to the hormone response element (homodimer formation may not be necessary for binding of estrogen to its hormone response element; Furlow *et al.*, 1993).

Phosphorylation of steroid receptors is also involved in activation, DNA binding, and activation of transcription. Hormone binding by steroid receptors is increased when tyrosine kinase is added to cultures of receptors. There are multiple sites for phosphorylation on most steroid receptors and hyperphosphorylation occurs after ligand binding. Serine is the primary amino acid phosphorylated, with minor amounts of threonine phosphorylated (this may be switched in the Er). Phosphorylation occurs in several domains, particularly the hormone binding and DNA binding domains and the hinge region. It has been proposed that phosphorylation must precede dimerization; however, this has not been proven. There are results which indicate that phosphorylation of serine/threonine in some domains inhibits normal steroid receptor function (see Orit *et al.*, 1992).

Hormone Response Elements

Hormone response elements are the nucleotide sequences recognized by the steroid receptors. Hormone response elements are sequences which have palindromic structure. For steroid hormones receptors, hormone response elements are unequal halves with three non-conserved base pairs between each of the halves. Like the steroid receptor super-family, the hormone response elements can be separated into subfamilies with the glucocorticoid/progesterone subfamily in one group and the estrogen subfamily in another. The consensus sequence for the glucocorticoid/progesterone subfamily half palindrome hormone response element is TGTYCT, while it is TGACC for the estrogen subfamily. The difference between these two elements is at the third position, this is a T in the glucocorticoid/progesterone family and an A in the Er family. The sequence responsible for determination of progesterone or Er binding is in the area between Cys 3 and 4 and the amino acid residues just downstream from that point in the first zinc finger (Mader *et al.*, 1989; Umesono and Evans, 1989). There are specific hormone response element recognition sites between Cys 5 and 6 located on the second finger as well. These may be important in dictating the orientation of the receptor dimers (Härd *et al.*, 1990).

Steroid hormones control the activity of various genes in target cells through the action of ligand activated

transcriptional modulators which act by binding to the hormone response element on the DNA. This interaction with the DNA-bound receptor with basal transcriptional machinery and sequence specific transcription factors mediate the transcriptional effects of the steroid hormone.

Estrous Cycle

The estrous cycle in the ewe is 16 to 17 days in length, but there are some minor variations in length, due to age and stress (Mckinzie and Terrill, 1937).

Estrous behavior occurs in most breeds of sheep during the fall and winter, while anestrus occurs during the spring and summer. This assures that birth of young occurs in the spring, when conditions for their survival are optimal. Unlike the estrous cycle, there is considerable variability in the duration of the breeding season among breeds. The breeding season is under photoperiodic control and those breeds originating from mild climates have much longer breeding seasons than those breeds which originated in more northern regions (Robinson, 1959).

Estrus is defined as the period of time when the ewe willingly allows the ram to mount. The first day a ewe will allow a ram to mount is, by convention, designated Day 0 of the estrous cycle. Estrous behavior generally lasts 24 to 48 h; however, there is a considerable variation in duration. Differences in duration of estrous behavior can

be attributed to several factors. The most studied, however, are the effects of ovulation rate and the ram. Breeds which typically have high ovulation rates exhibit a longer period of estrus (Bindon *et al.*, 1979). Continuous presence of a ram reduces length of estrus, as compared to that for ewes that experience intermittent exposure to a ram (Parsons, 1967). Regardless of the duration of estrus, ovulation occurs about 30 h after the onset of estrous behavior in response to the ovulatory surge of LH (Mckinzie and Terrill, 1937).

The two ovarian steroids primarily involved in the estrous cycle are progesterone and estradiol. Progesterone is secreted by the CL, while estradiol is secreted by the follicles (Short *et al.*, 1963). The CL is made up of two steroidogenic cell types distinguished by size and function. The large luteal cells are believed to be of granulosa cell origin and the small luteal cells appear to be of theca cell origin (Meidan *et al.*, 1990). Large luteal cells secrete oxytocin (Rodgers *et al.*, 1983) and spontaneously secrete large amounts of progesterone (Hulet and Shelton, 1980). Conversely, small luteal cells must be stimulated by LH to produce progesterone (Fitz *et al.*, 1884). Small luteal cells have high numbers of LH receptors and very low numbers of $PGF_{2\alpha}$ receptors. In contrast, large luteal cells have abundant $PGF_{2\alpha}$ receptors and few LH receptors (Alila *et al.*, 1988). The preponderance of $PGF_{2\alpha}$ receptors on large luteal

cells may explain why they are affected first during luteolysis, resulting in a rapid decrease in progesterone secretion at the onset of luteolysis (Branden *et al.*, 1988). $PGF_{2\alpha}$ activates the protein kinase C (PKC) system in large luteal cells, which leads to inhibition of progesterone synthesis. Although activation of the PKC system in small luteal cells also inhibits progesterone synthesis, it is not clear what hormone is involved in the activation of PKC in these cells (Niswender and Nett, 1994).

Progesterone levels are nearly undetectable from Day 0 to Day 3 and then increase gradually until Day 8. The levels remain constant, thereafter, ranging from 1.5 to 3 ng/ml (Bindon *et al.*, 1979), until Day 15-16 when progesterone decreases rapidly in blood to below 1 ng/ml, and the next cycle commences (Stabenfeldt *et al.*, 1969). While there is very little difference in temporal changes in progesterone secretion by CL, differences have been reported for maximal circulating concentrations of progesterone across breeds. Progesterone concentrations are generally higher at mid-cycle in those breeds with higher ovulation rates compared to breeds with lower ovulation rates (Bindon *et al.*, 1979). Progesterone concentrations are also higher in the middle of the breeding season, compared to the beginning or the end of the season (Legan *et al.*, 1985).

Progesterone levels begin to fall even before CL regression becomes morphologically apparent and luteolysis

is initiated by $PGF_{2\alpha}$ release from the non-gravid uterus. Pulsatile secretion of $PGF_{2\alpha}$ is first apparent about Day 15 of the cycle and continues to increase until estrus. The role of $PGF_{2\alpha}$ in luteolysis and the attenuation of its production will be discussed in more detail in the sections on luteolysis and maternal recognition of pregnancy.

Plasma estradiol concentrations begin to increase just after the initial decrease in progesterone is noted. Estrogen concentrations in the plasma continue to increase from basal levels of 1 pg/ml to highest levels of about 10 pg/ml (Baird *et al.*, 1976) at the LH surge, after which time estrogen levels decline rapidly and remain at basal levels through the luteal phase except for small increases associated with follicular waves. Estradiol is produced by rapidly growing preovulatory follicles during proestrus (Days 15-16).

Both progesterone and estradiol are secreted in pulses. Progesterone, however, does not follow a pattern set by gonadotropins as does estradiol. Pulses of estradiol precede pulses of LH and both pulse frequency and amplitude of estradiol increase during the follicular phase (Baird, 1978).

LH is secreted episodically, in the ewe, and varies in both frequency and amplitude throughout the estrous cycle. The basal level of LH secretion (~1 ng/ml) is required for

CL function and steroidogenesis, while the LH surge initiates ovulation and CL formation (Goding *et al.*, 1970).

The LH surge is characterized by a dramatic rise in LH pulse frequency a few hours prior to ovulation after which LH returns to basal levels. On average the entire ovulatory surge of LH occurs within 12 h, and coincides with the onset of estrus. This is followed by ovulation about 30 h later. The CL begins to produce progesterone by Day 3 and the recurring estrous cycles continue during the breeding season, until ewes become pregnant, or enter anestrus.

Changes in the secretion of LH during the normal cycle and ovulation are tightly controlled by changes in the generation of GnRH pulses (reviewed in Goodman, 1994). During the early luteal phase, tonic LH secretion decreases because the increasing amounts of progesterone, along with estradiol, suppress the activity of the GnRH neural oscillator and decrease LH pulse frequency. Tonic LH levels then remain low as long as progesterone levels are increased which attenuates GnRH pulse frequency. When progesterone levels fall following luteolysis, the frequency of the GnRH pulse generator increases, which may in turn stimulate GnRH receptor gene expression and increased expression of GnRH receptors (Turzillo *et al.*, 1995). Increases in GnRH pulse generation then serve to increase the pulse frequency of LH and accounts for the rise in LH secretion during the follicular phase of the sheep.

Endometrial Steroid Receptors

It is clear that the uterine environment is dynamic and ever changing. The changes that occur throughout the estrous cycle with regard to both physiological and biochemical activities are controlled by both changing steroid hormone profiles and changes in expression of their receptors. While finite control of uterine steroid receptors is not fully understood, changes in Er and Pr dynamics throughout the estrous cycle and pregnancy are being examined.

In the cyclic ewe, the Er is generally abundant just before and after estrus (Wathes and Hamon, 1993). Numbers of Er fall dramatically from Day 3, to a low on Days 10 to 14, followed by a dramatic increase beginning on Day 14, to peak values at estrus (Koligian and Stormshak, 1976; Miller *et al.*, 1977; Zelinski *et al.*, 1980; Cherny *et al.*, 1991; Ott *et al.*, 1993b). Steady state levels of endometrial Er mRNA are highest on Day 1 of cyclic ewes, decline between Days 1 and 6, and increase between Days 11 and 15 (Spencer and Bazer, 1995). Myometrial Er mRNA in these animals was also highest on Day 1, but decreased to Day 6 and remained low thereafter. Cherny *et al.* (1991) reported that endometrial Er regulation was not homogenous throughout endometrial tissue, but varied by tissue type (caruncular or intracaruncular), as well as cell type (epithelial, stromal or glandular), and that steroidogenic control within tissues

was also variable. Spencer and Bazer (1995) have further demonstrated, using in situ hybridization and immunocytochemical approaches, that distinct and tissue- and cell-specific alterations in uterine Er and Pr mRNA and protein expression during the estrous cycle of the ewe generally paralleled the overall changes noted in steady state levels of Er and Pr mRNAs. In the endometrium, Pr mRNA and protein expression disappeared from the luminal and shallow glandular epithelium between Days 6 and 13, whereas Er mRNA and protein expression was low on Days 6 and 11 and increased between Days 11 and 15 in the luminal and shallow glandular epithelium. Er mRNA and protein were consistently present at low levels in the stroma and deep glandular epithelium. Progesterone is inhibitory to Er formation in several species (Brenner *et al.*, 1974; Hsueh *et al.*, 1975 and 1976; Tseng and Gurpide, 1975; West *et al.*, 1976; Bhakoo and Katzenellenbogen, 1977; Spencer *et al.*, 1995a; 1995b) including sheep (Koligian and Stormshak, 1977b; Zelinski *et al.*, 1980; Cherny *et al.*, 1991; Spencer *et al.*, 1995a; 1995b). Estrogen, on the other hand, is stimulatory to it's own receptor formation (Anderson *et al.*, 1975; Bhakoo and Katzenellenbogen, 1977; Zelinski *et al.*, 1980; Cherny *et al.*, 1991). Control of Er, however, is not a simple matter and probably involves several factors which affect each of the uterine cell populations in various ways (Cherny *et al.*, 1991).

The Pr is most prevalent in ovine endometrium around estrus (Miller *et al.*, 1977; Zelinski *et al.*, 1980; Ott *et al.*, 1993b) with the highest concentrations of receptors reported on Day 2 (Miller *et al.*, 1977) and the lowest from Days 10 to 14. In cyclic ewes, endometrial Pr mRNA levels are highest on Day 1, decrease between Days 1 and 11, and then increase between Days 13 and 15. Myometrial Pr mRNA levels are highest on Day 1 and decline thereafter (Spencer and Bazer, 1995). Estrogen appears to be the primary stimulus for Pr formation (Milgrom *et al.*, 1973; Leavitt *et al.*, 1974; Zelinski *et al.*, 1980; Aronica and Katzenellenbogen, 1991). Progesterone, however, can exert both positive and negative influences on uterine Pr expression. Progesterone in cyclic animals is clearly inhibitory to endometrial Pr expression, probably through down-regulation of Pr (Milgrom *et al.*, 1973; Leavitt *et al.*, 1974; Vu Hai *et al.*, 1977). Progesterone down-regulation of its own receptor removes the progesterone block (McCracken *et al.*, 1984), allowing Er up-regulation, and formation of OTr which, when bound by oxytocin, initiates pulsatile release of $PGF_{2\alpha}$. In pregnant ewes it had been assumed that Pr are maintained in the presence of high progesterone for extended periods (Ogle *et al.*, 1989, 1990; Ott *et al.*, 1993b; Mirando *et al.*, 1993). In the pregnant ewe this was suggested to be achieved by stabilization of the Pr by a product of the conceptus (Ott *et al.*, 1993b). However,

others have shown that continuous exposure of the endometrium to progesterone down-regulates endometrial Pr mRNA and protein abundance in the luminal epithelium, shallow glandular epithelium, and stroma (Wathes and Hamon, 1993; Spencer and Bazer, 1995; Spencer *et al.*, 1995b). The mechanism responsible for this is currently poorly understood but may involve Pr-mediated decreases in Pr gene transcription (Alexander *et al.*, 1989; Read *et al.*, 1988). Results from studies performed by Spencer *et al.* (1995b) have shown that negative regulation of the Pr gene in the endometrial epithelium occurs in both cyclic and pregnant ewes, because Pr mRNA abundance and immunoreactive Pr protein declined in the endometrial luminal epithelium and shallow glandular epithelium after Day 6. Thus, the current hypotheses is that pregnancy does not stabilize or up-regulate Pr gene expression in the endometrium.

Luteolysis

Luteolysis is initiated in ruminants in response to the pulsatile release of by $PGF_{2\alpha}$ produced by the uterus (McCracken *et al.*, 1981; Hooper *et al.*, 1986). $PGF_{2\alpha}$ is released from the uterus in the nonpregnant ewe in a series of 5-8 episodes (Thornburn *et al.*, 1973) with 6-8 h between each series. McCracken *et al.* (1984) have shown that the CL must be exposed to approximately 5 pulses of PGF over a 25-hour period to undergo complete luteolysis. These episodes

begin just prior to the onset of luteal regression. At this time the progesterone level has not begun to decline (Zarco *et al.*, 1988b) but Pr are very low which allows expression of Er and OTr. The pulsatile secretion of $PGF_{2\alpha}$ is initiated by the secretion of oxytocin from the posterior pituitary and is escalated by oxytocin of luteal origin. The result is a synchronous pulsatile release of oxytocin from the CL on each ovary and from the posterior pituitary in ewes (Hooper *et al.*, 1986). Oxytocin from the CL and $PGF_{2\alpha}$ from the uterus act together in a positive feedback loop to generate pulses of $PGF_{2\alpha}$ required for luteolysis.

Oxytocin is released from the posterior pituitary (Hooper *et al.*, 1986) as well as the CL where it is produced by large luteal cells (Rodgers *et al.*, 1983; Wathes and Denning-Kendal, 1992) and is secreted into the ovarian vein (Wathes and Swann, 1982; Flint and Sheldirck, 1982; Flint and Sheldrick, 1986). In large luteal cells, the oxytocin gene is transcribed on Days 0-4 (Jones and Flint, 1986). Oxytocin mRNA is translated into oxytocin from Days 4-7. Stores of oxytocin and its neurophysin in luteal cells are highest on Days 10-12 (Silvia *et al.*, 1991; Rhodes and Nathanielsz, 1990). Flint and Sheldrick (1983) reported that the rise and fall of oxytocin followed that of progesterone, with the lowest levels noted at the time of ovulation. However, there is no indication that progesterone directly affects the synthesis of oxytocin.

There is considerable evidence, however, for the association of oxytocin and $PGF_{2\alpha}$, in the formation of a positive feedback loop to increase pulsatile release of luteolytic $PGF_{2\alpha}$.

As stated earlier, there is a positive feedback loop between oxytocin and $PGF_{2\alpha}$. That is, $PGF_{2\alpha}$ can stimulate luteal oxytocin secretion and oxytocin can stimulate endometrial $PGF_{2\alpha}$ secretion. There is evidence that the initiation of this loop is with $PGF_{2\alpha}$ secretion from the uterus due to the fact that $PGF_{2\alpha}$ release occurs prior to pulsatile release of luteal oxytocin (Moore *et al.*, 1986). While it is possible that the uterus has an endogenous clock, possibly estrogen, that triggers the release of the initial $PGF_{2\alpha}$ secretion, pituitary oxytocin is the more accepted mediator of the initial uterine $PGF_{2\alpha}$ secretion which in turn begins the $PGF_{2\alpha}$/oxytocin feedback loop (see Silvia *et al.*, 1991; McCracken *et al.*, 1991).

Once the $PGF_{2\alpha}$/oxytocin loop is established, it serves to increase $PGF_{2\alpha}$ concentrations in the ovarian pedicle. Increasing concentrations of $PGF_{2\alpha}$ binding to their receptors on the large luteal cells, cause a reduction in viable cell numbers *in vitro*. Several different mechanisms have been put forth to explain the luteolytic effects of $PGF_{2\alpha}$ (see Niswender and Nett, 1994; Spencer and Bazer, 1995). These mechanisms include: 1) a rapid decrease in luteal blood flow; 2) a reduction in the number of LH

receptors and/or an uncoupling of the LH receptor from adenylate cyclase; 3) activation of protein kinase C; 4) influx of high levels of calcium; and 5) a cytotoxic effect (Silvia *et al.*, 1984a). Luteolysis does not require withdrawal of basal LH support, but PGF may activate protein kinase C in large luteal cells to inhibit progesterone production (Wiltbank *et al.*, 1991) and cause luteolysis (McGuire *et al.*, 1991). Treatment of large luteal cells with PGF increases intracellular calcium (Wiltbank *et al.*, 1989), which appears to mediate the cytotoxic effects of $PGF_{2\alpha}$ probably through typical apoptotic changes (Sawyer *et al.*, 1990) as well as decrease expression of mRNA for 3β-hydroxysteroid dehydrogenase (Hawkins *et al.*, 1993).

Effects of Ovarian Steroids on Prostaglandin-$F_{2\alpha}$ Secretion

There is a great deal of information on the effects of steroids on $PGF_{2\alpha}$ formation and/or luteolysis. It is obvious that progesterone and estrogen affect $PGF_{2\alpha}$ production and secretion, and are, therefore, important steroids affecting luteolysis.

Progesterone affects

One of the most compelling studies to link progesterone with normal cyclical estrus activity in ewes was that of French and Spennetta (1981) who showed that immunization of ewes against progesterone resulted in erratic estrous cycles. Progesterone initiates $PGF_{2\alpha}$ secretion in

ovariectomized ewes after 7 days of treatment, but prior to progesterone treatment, $PGF_{2\alpha}$ secretion was minimal (Scaramuzzi *et al.*, 1977). Prostaglandin-$F_{2\alpha}$ secretion can also be induced early in the cycle (up to 32 h post-estrus) by progesterone treatment (Ginther, 1969; Ottobre *et al.*, 1980). Administration of exogenous progesterone to cyclic ewes during metestrus decreases interestrous intervals, while administration of the Pr antagonist RU486 during the early luteal phase delays the onset of endometrial PGF production and luteolysis (Morgan *et al.*, 1993). Apparently the uterus requires the influence of progesterone for a period of 10 to 12 days to produce luteolytic $PGF_{2\alpha}$ in a normal cyclic manner (Vallet *et al.*, 1990).

Progesterone is also necessary to elicit physiological changes in uterine responsiveness noted throughout the cycle. Humanics and Silvia (1988) utilized ovariectomized ewes to show this effect. Ewes were first pretreated with a hormonal regime that would mimic that of the steroid pattern noted in cyclic animals 6 days prior to estrus. Ewes were then treated with progesterone for 15 days and responsiveness to oxytocin (as determined by PGFM response) was measured on Days 5, 10 and 15 of treatment. They found that there was no PGFM response until Day 15. Vallet *et al.* (1990) used a similar model in which a subset of ewes received no steroid pretreatment and then progesterone alone

for 12 days to cause the uterus to become responsive to oxytocin challenge.

There are several ways that progesterone changes the endometrial milieu that could explain changes in uterine responsiveness to oxytocin. Brinsfield and Hawk (1973) reported that the accumulation of lipid droplets in uterine epithelium of ewes were induced by progesterone. In rats progesterone induces both phospholipid and triglyceride accumulation (Mamimekalai *et al.*, 1979 ;Boshier *et al.*, 1981). Progesterone has been shown to affect several factors associated with $PGF_{2\alpha}$ metabolism. Raw and Silvia (1991) have shown that progesterone treatment of ovariectomized ewes for 16 days resulted in increased phospholipase-C activity. Phospholipase-C activity has been found to increase during the period of luteolysis in the ewe as well (Silvia and Raw, 1993). While there is no doubt that progesterone has an effect on phospholipase-C activity, Silvia and Raw (1993) suggest that control of $PGF_{2\alpha}$ metabolism may occur later in the synthesis pathway.

Progesterone has an effect on the prostaglandin synthetase enzyme in cattle, and ovariectomized ewes treated with progesterone also respond with elevated uterine endometrial prostaglandin-H synthetase activity (Raw *et al.*, 1988). Eggleston *et al.* (1990) reported that prostaglandin-H synthetase mRNA increased in intact ewes treated with progesterone early in the cycle. There was also an

increased incidence of early luteolysis in these ewes. An increase in $PGF_{2\alpha}$ release also occurs when progesterone is removed at a time corresponding to normal progesterone decline in cyclic ewes (Leavitt *et al.*, 1985). Therefore, progesterone may stimulate $PGF_{2\alpha}$ secretion early in the cycle, but exerts an inhibitory effect late in the cycle (see Silvia *et al.*, 1991).

Estrogen affects

Ford *et al.* (1975) showed that pharmacological levels of estradiol (two injections given 12 h apart) resulted in an increase in $PGF_{2\alpha}$ within 12 h after the second injection. This effect was the same for both control and progesterone primed ewes. McCracken (1980) used ovariectomized ewes, and Sharma and Fitizpatrick (1974) used anestrus ewes, to elicit a $PGF_{2\alpha}$ response to estradiol in as little as 6 h. Results of these studies indicate that in ovariectomized ewes, at least, there is no requirement for progesterone in $PGF_{2\alpha}$ secretion. However, in studies in which physiological levels of estradiol were administered to ovariectomized ewes, estradiol alone was less effective in eliciting a $PGF_{2\alpha}$ response (Homantics and Silvia, 1988). In studies of intact ewes, estrogen was also ineffective in eliciting a $PGF_{2\alpha}$ response when administered alone. In a study in which the uterus was auto-transplanted to the neck of the ewe, Scaramuzzi *et al.* (1977) found that progesterone pretreatment was required to get a full $PGF_{2\alpha}$ response. In

ewes which received estradiol alone there was a slight, but nonsignificant, increase in the $PGF_{2\alpha}$ response. Endometrium from ewes treated with estradiol for several days *in vivo* were also unable to respond with $PGF_{2\alpha}$ secretion *in vitro* (Findly *et al.*, 1981; Raw and Silvia, 1991).

Effects of estrogens on $PGF_{2\alpha}$ release are much greater when estrogen is administered in conjunction with progesterone or in a regime that mimics the steroidogenic patterns of the normal estrous cycle. In cyclic ewes, estradiol injections on Days 11 and 12 (in one study), or Day 10 (in another), were able to induce luteal regression (Stormshak, 1969; Cook *et al.*, 1974). In a similar study, Hawk and Bolt (1970) treated a total of 94 ewes with estradiol on two successive days with groups beginning treatment on Days 1 through 11. They found that estradiol had no effect on CL weights until Day 10 of the cycle. If ewes were not treated with estradiol until Days 11 and 12 CL weights were decreased by Day 15. Secretion of $PGF_{2\alpha}$ is also enhanced when ewes are treated with estrogen and progesterone (Ford *et at.*, 1975). Hixon and Flint (1987) showed that inositol phosphate metabolism as well as $PGF_{2\alpha}$ secretion increased when estradiol was administered at mid-cycle (Days 9 and 10). Barcikowski *et al.* (1974) concluded that the $PGF_{2\alpha}$ response was due to a direct effect of estrogen on the uterus when physiological quantities of estradiol were infused either into the uterine artery or

systemically. Only those ewes which received treatment locally (at the uterus), and late in the cycle responded with elevated secretion of $PGF_{2\alpha}$. Ovariectomized ewes treated with progesterone for at least 5 days also responded with greater $PGF_{2\alpha}$ secretion than ewes treated with estrogen alone (Scaramuzzi *et al.*, 1977). As discussed earlier Vallet *et al.* (1990) reported that progesterone alone will elicit a $PGF_{2\alpha}$ response when ewes are challenged with oxytocin. This study, however, showed that a combined treatment of progesterone and estrogen resulted in the highest response to oxytocin challenge.

Ovariectomized ewes, which have low basal concentrations of both progesterone and estradiol, have also been used to study the factors which control oxytocin-induced $PGF_{2\alpha}$ release (Beard and Lamming, 1994; Beard *et al.*, 1994). In these ewes, expression of the endometrial OTr was constitutively present in high levels in luminal and superficial glandular epithelium, and treatment with progesterone initially caused a complete loss of OTr expression (Wathes and Lamming, 1995; Stevenson *et al.*, 1994; Sheldrick and Flint, 1985). However, chronic progesterone treatment for 12 days resulted in an increase in endometrial phospholipid stores, OTr expression, prostaglandin synthase activity, and oxytocin-induced pulsatile release of PGF (Salamonsen, 1992; Spencer *et al.*, 1995b; Vallet *et al.*, 1990). The OTr that develop as

progesterone down-regulates Pr are localized exclusively to luminal and superficial glandular epithelium, which is essentially identical to the spatial expression of OTr during luteolysis (Wathes and Lamming, 1995).

Estradiol, like progesterone, affects the enzymes involved in the synthesis of $PGF_{2\alpha}$. Estrogen treatment on Days 9 and 10 of the cycle results in increased inositol phosphate metabolism (an indication of phospholipase-C activity) within 12 h, over that of controls. However, in ovariectomized ewes which received physiological doses of steroids for 16 days, there was a decrease in phospholipase-C activity in ewes treated with estrogen alone, or estrogen plus progesterone (Raw and Silvia, 1991). Prostaglandin synthetase mRNA was decreased in ovariectomized ewes treated with estrogen alone, however, prostaglandin synthetase mRNA was not different from controls when ewes were treated with progesterone and estrogen (Salamonsen *et al.*, 1991). In this study, prostaglandin synthetase mRNA was decreased, but there was no difference in the levels of immunoreactive prostaglandin synthetase in luminal and glandular epithelial cells of ewes that received progesterone or progesterone plus estrogen.

Summary

The results of these studies clearly indicate that there is an effect of the ovarian steroids on $PGF_{2\alpha}$ synthesis and secretion. While this effect is apparent

after progesterone treatment alone, estrogen appears to facilitate the complete response in the normal, cyclic ewe.

Endometrial Oxytocin Receptor

The release of $PGF_{2\alpha}$ is initiated by oxytocin after release from large luteal cells (Rodgers *et al.*, 1983), and binding to its endometrial receptor (Flint and Sheldrick, 1983; Roberts *et al.*, 1976). The appearance of OTr on endometrial epithelium (surface primarily) about Day 14 of the cycle is believed to set up the oxytocin/$PGF_{2\alpha}$ feedback loop which initiates and insures luteolysis. Pulses of $PGF_{2\alpha}$ have been shown to coincide with pulses of plasma oxytocin (Flint and Sheldrick, 1983; Hooper *et al.*, 1987). The binding of oxytocin initiates the phosphoinositide-phospholipase-C transduction signal, one action of which is to mobilize arachidonic acid, which results in the formation of $PGF_{2\alpha}$.

Endometrial OTr concentrations rise rapidly 48 h prior to estrus (Sheldrick and Flint, 1985). Endometrial OTr begin to increase on Day 14, reach maximum values at estrus and decline by Day 5 of the subsequent cycle. This is coincidental with the decrease in plasma progesterone, Pr, the rise in estrogen and Er (Sheldrick and Flint, 1985; Wallace *et al.*, 1991) during proestrus. Through the use of receptor binding studies it was shown that endometrial OTr are more concentrated in the intercaruncular than in the

caruncular tissue of ewes in estrus, while Days 13 through 15 there were more receptors located in caruncular tissues (Shledrick and Flint, 1985). Autoradiographic studies have shown that at the time of luteolysis, endometrial OTr are found only on the luminal epithelial cells (Ayad *et al.*, 1991a, 1991b; Wallace *et al.*, 1991). The appearance of receptors in stromal tissues and glandular epithelial cells occurs after luteolysis. It is proposed that the apparent differential regulation of these receptor populations may be important in the process of luteolysis (or lack of luteolysis in the pregnant ewe).

The effect of oxytocin on its own receptor was examined by Flint and Sheldrick (primarily for therapeutic purposes; 1985). In this study, systemic infusion of oxytocin resulted in a lengthening of the interestrous interval. Upon examination there was no apparent lysis of luteal tissues. It was proposed by others that the action of oxytocin was to down-regulate its own receptor and that the increase in endometrial OTr at the time of luteolysis was due to removal of this down-regulation by decreasing concentrations of oxytocin in plasma. However, since plasma oxytocin concentrations decrease prior to this time, this seems unlikely. Down-regulation was also proposed as a possible explanation for the lack of OTr in pregnant ewes in response to oxytocin produced by the conceptus (Lacroix *et al.*, 1988). This does not appear likely since ovine

trophoblast cells do not express oxytocin (Parkinson *et al.*, 1991) and intrauterine infusions of oxytocin have no effect on cycle length or endometrial OTr expression (Parkinson *et al.*, 1991; Ayad *et al.*, 1993). It might be argued that uterine proteases denatured the infused oxytocin and this was the reason for the difference in cycle extension noted between these studies. However, OTr expression has been shown to be absent on the luminal epithelium if oxytocin was infused into the uterine lumen (Ayad *et al.*, 1993). Thus, OTr may be localized to the basolateral domain of the epithelial cells so that intraluminal oxytocin may not have access to those receptors. Since the conceptus does not express oxytocin (Parkinson *et al.*, 1991), down regulation does not appear to be a controlling factor in OTr concentration at the time of maternal recognition of pregnancy. The fact that endometrial OTr concentrations are highest when plasma oxytocin concentrations are low (Flint and Sheldrick, 1985) indicates that oxytocin effects are exerted at low receptor occupancy and suggests that control of uterine responsiveness to oxytocin is through control of OTr gene expression, rather than control of oxytocin secretion.

Oxytocin receptors are clearly under steroid hormone control. Uterine OTr increase (in rodents) in response to estrogen (Soloff, 1975). As indicated by $PGF_{2\alpha}$ secretion, prolonged progesterone treatment followed by estrogen

stimulation resulted in maximum OTr formation (McCracken *et al.*, 1981). It was proposed (using a hamster model) that estrogen stimulates endometrial OTr formation and that progesterone exerts inhibitory actions through progesterone-induced inhibitors which block estrogen binding to its receptor (Okulicz *et al.*, 1981). These findings led McCracken *et al.* (1984) to propose that there was a progesterone block to uterine OTr formation during Days 5 to 14 of the cycle in ewes. At the end of this period the endometrium became refractory to the progesterone block, probably because of down-regulation of Pr by progesterone. Removal of the progesterone block, along with the stimulatory influence of rising estrogen, caused up-regulation of endometrial OTr. Vallet *et al.* (1990) found that endometrial OTr were high in ovariectomized ewes, and treatment with progesterone initially caused a complete loss of OTr expression (Wathes and Lamming, 1995; Vallet *et al.*, 1990; Lau *et al.*, 1992) which indicates that the role of ovarian steroids, in control of endometrial OTr, is inhibitory. Interestingly, these receptors were apparently uncoupled from $PGF_{2\alpha}$ synthesis as noted by the lack of PGFM response after challenge with exogenous oxytocin. Since progesterone increases phospholipid stores (Boshier *et al.*, 1987) and prostaglandin synthase activity is necessary for conversion of arachidonic acid to $PGF_{2\alpha}$ (Eggleston *et al.*, 1990) this "uncoupling" phenomenon was apparently related

to a reduction in the machinery necessary for the increased PGF synthesis. Progesterone treatment alone, for a period of 12 days, was sufficient to increase endometrial phospholipid stores, OTr expression, prostaglandin synthase activity, and oxytocin-induced pulsatile release of $PGF_{2\alpha}$ (Salmonsen, 1992; Spencer *et al.*, 1995b; Vallet *et al.*, 1990). Treatment with progesterone for 5 days was not sufficient to stimulate endometrial OTr formation. A regime meant to closely mimic a normal estrous cycle (progesterone pretreatment, estradiol, progesterone for 12 days, and estradiol on Days 11 and 12 of progesterone treatment), resulted in only a slight increase in OTr number over that of progesterone alone. Estradiol alone was inhibitory to receptor formation. It was proposed by these authors that formation of endometrial OTr was under the inhibitory control of progesterone alone. Also, estrogen's action may be biphasic, in that estrogen may be stimulatory on Days 1 through 2 then inhibitory on Days 5 through 7. Similar findings were reported in studies utilizing ovariectomized ewes (Lamming *et al.*, 1991; Zang *et al.*, 1992; Lau *et al.*, 1992; Lau *et al.*, 1993) and uterine explant tissues (Sheldrick and Flick-Smith, 1993). The overall effects of estrogen on the timing, magnitude and pattern of $PGF_{2\alpha}$ response to oxytocin may be mediated through increases in OTr gene expression (Beard and Lamming, 1994; McCrackin *et al.*, 1984), enhanced coupling of OTr to its second messenger

signal transduction system (Bouvier *et al.*, 1991) and increased activity of the machinery which drives prostaglandin synthase activity (Eggleston *et al.*, 1990; Huslig *et al.*, 1997).

Maternal Recognition Of Pregnancy

The term "maternal recognition of pregnancy" was first used in 1969 in a review published by R.V. Short. However, it had been known for some time that the conceptus in some way affected CL life-span. This was first alluded to in 1945 by Casida and Warwick who found that CL were maintained in pregnant ewes and that the conceptus was dependent on the CL for continued development until Day 55 of gestation. It was not until embryo transfer studies showed that synchronous transfers (Day 12 embryo into a Day 12 uterus) were successful, but embryo transfers on Day 13 were not, that the conceptus was directly implicated as having antiluteolytic effects. To determine that the Day 13 conceptus was not affected by the transfer procedure and that this was the reason there was not cycle extension in these ewes, Day 13 conceptuses were transferred to Day 12 cyclic sheep and the result was extension of the cycle. These results showed there was some affect of the conceptus that had to occur on Day 12 to prevent luteal regression in the pregnant ewe (Moor and Rowson, 1964; 1966a). A similar

study also detected the antiluteolytic effect of the ovine conceptus (Niswender and Dziuk, 1966).

To further examine the antiluteolytic effect of the conceptus, Moor and Rowson (1966b) removed conceptuses on Days 5 through 15 of pregnancy. They found that removal of the conceptus on Day 12 or before resulted in a cycle length that was normal (~17 days). However, if the conceptus was removed on Day 13 or after there was an extension in the life-span of the CL on average to Day 25. This study clearly indicated that the conceptus was affecting CL life-span, and that this effect occurred on Day 12 of pregnancy. Since it was known that the uterus-initiated CL regression (see Luteolysis section) and that this was a local affect, Moor and Rowson (1966c) questioned whether the effect of the conceptus was also local. The authors found when conceptuses were transferred to an isolated ipsilateral horn the CL was maintained, but when the transfer was to an isolated contralateral horn the CL regressed. However, if embryo transfers to the contralateral horn coincided with removal of the ipsilateral horn the CL was maintained. These studies indicated that the conceptus exerts a local unilateral antiluteolytic effect. This was further indicated by the fact that embryo transfers to one isolated horn of a uterus in ewes with a CL on each ovary resulted in maintenance of the ipsilateral CL, but not the contralateral CL. Taken together, these studies clearly indicate that the

conceptus overcomes the local luteolytic effect of the uterus.

The next question was how does the conceptus exert its antiluteolytic effect. To examine this, Day 14 and Day 15 conceptus were collected, homogenized and frozen/thawed or heat treated. These homogenates were infused into the uterine lumen on Day 12 or daily for the treatment period. Infusions of conceptus homogenates on Day 12 alone or of heat-treated conceptus homogenates were not effective in extending the estrous cycle. Repeated daily infusions of frozen/thawed conceptus homogenates extended the cycle, on average to 22 days. Infusion of Day 25 sheep conceptuses homogenates or Day 14 pig homogenates had no effect on cycle length (Rowson and Moor; 1967). These studies indicated that the antiluteolytic effect was apparently species specific and time dependent. Furthermore, the fact that the antiluteolytic effect was present in frozen/thawed, but not heat-treated conceptus homogenates led the authors to suggest that the responsible factor was chemical in nature and heat labile. Similar studies indicated that there was an antiluteolytic effect of intrauterine infusion of bovine conceptus homogenates in the cow (Northly and French, 1980).

McCracken's report (1971) that $PGF_{2\alpha}$ is released into the uterine vein by endometrial tissues and that the timing of this release coincides with CL regression, initiated a great deal of interest in the effect of the conceptus on

$PGF_{2\alpha}$ release. Moore and Watkins (1982) found that on Days 12 and 13, cyclic ewes responded with a pulse of PGFM for each pulse of oxytocin neurophysin and that this effect was absent in pregnant ewes. Pratt *et al.* (1977) reported that when $PGF_{2\alpha}$ was injected into the largest follicle on the ovary bearing the CL of cyclic and pregnant ewes the cyclic ewes had a 1.5 day shorter interestrous interval, while none of the pregnant ewes returned to estrus. In this same report, intrauterine infusions of prostaglandin-E into pregnant ewes resulted in longer cycle lengths than for control ewes. Silvia and Niswender (1984) found that less $PGF_{2\alpha}$ (4 mg/58 kg body weight as compared to 10 mg/58 kg) was required to cause luteolysis in cyclic compared to pregnant ewes. In further studies by these same authors, the protective properties the conceptus bestowed on the CL, in the presence of $PGF_{2\alpha}$ challenge, did not occur until Day 13 and was lost by Day 26. Similar results were found when ewes were challenged with oxytocin (Fairclough *et al.*, 1984; Silvia *et al.*, 1992). Estradiol treatment also induces luteolysis in cyclic, but not pregnant ewes (Kittok and Britt, 1977), indicating an affect of estradiol in $PGF_{2\alpha}$ production which the conceptus is able to block.

McCracken (1984) first proposed that estradiol-induced formation of endometrial OTr, which bound oxytocin, of pituitary or luteal origin, to induce pulsatile release of $PGF_{2\alpha}$. It was this process that began the feedback loop

which resulted in luteolysis (see Luteolysis section). Sheldrick and Flint (1985) reported that in pregnant ewes, the increase in OTr numbers detected in proestrus of cyclic ewes was attenuated. Oxytocin receptor expression is high from Day 14 of one cycle to Day 2 of the next cycle, but is almost completely absent in caruncular and intracaruncular endometrium of pregnant ewes (Flint and Sheldrick, 1986). Similar differences were found between cyclic and pregnant cows on Day 17 (Jenner *et al.*, 1991). Autoradiological studies of endometrial receptors determined that labeled oxytocin was concentrated in luminal epithelium, glandular epithelia and caruncular stroma of Day 15 cyclic ewes. However, in pregnant ewes there was no labeling of endometrial tissues (Ayad *et al.*, 1993).

Intrauterine injections of conceptus homogenates increase the interestrous interval in ewes (Rowson and Moor, 1967). Since the conceptus was directly affecting the maternal environment it was believed that this effect was through a secretory product. To determine if conceptus-conditioned culture media contained a pregnancy recognition factor, culture medium from incubations of Day 15 and 16 conceptuses were injected into the uterine lumen of cyclic ewes from Days 12 to 18 of the estrus cycle (Godkin *et al.*, 1984b). The CL life-span was prolonged in all ewes treated with oCSP based on maintenance of progesterone secretion and CL which had previously been marked with India ink. One

treated ewe maintained a functional CL until Day 52 when the project was terminated. In comparison, all the ewes treated with SP, as controls, ovulated by Day 25 when CL were checked at hysterectomy, and progesterone secretion had fallen by Day 19. This was the first study to conclusively show that oCSP could prolong CL life-span. Similar findings were reported by Vallet *et al.* (1988).

To determine the effect of oCSPs on uterine $PGF_{2\alpha}$ responsiveness after estradiol or oxytocin challenge, oCSP or plasma proteins were infused into the uterine lumen of ewes from Day 12 to Day 14 of the cycle (Fincher *et al.*, 1986). On Day 14 all ewes were challenged with estradiol. Jugular blood samples were drawn hourly over a 10 hour period and assayed for PGFM. On Day 15 one-half of the ewes from each treatment group were challenged with oxytocin or saline. Blood was drawn for PGFM analysis. The PGFM response was lower in ewes which received oCSP, compared to SP-treated controls, after estradiol and oxytocin challenge. These results indicated that the factor in oCSP which affects the endometrium is a secretory protein produced by the conceptus and that it can induce changes in the uterine environment which prevent normal cyclic responsiveness to estradiol and oxytocin. Vallet *et al.* (1988) and Mirando *et al.* (1990a) also reported that $PGF_{2\alpha}$ secretion after challenges with estradiol or oxytocin, was reduced significantly in oCSP-treated ewes.

The lack of $PGF_{2\alpha}$ responsiveness has been shown in the pregnant animal to be due to low oxytocin (Flint and Sheldrick, 1986) and estrogen (Findlay *et al.*, 1982) receptors on the luminal epithelium. An indirect measure of the OTr can be obtained by measurement of IP which is a factor in the signal transduction pathway stimulated by activation of the OTr (Flint *et al.*, 1986; Hixon *et al.*, 1987). Mirando *et al.* (1990a, 1990b) reported that in Day 16 pregnant ewes there was an attenuation of IP hydrolysis within endometrial tissues after *in vitro* stimulation with oxytocin, while in Day 16 cyclic ewes there was an increase in IP hydrolysis. The pregnant ewes also had high plasma progesterone concentrations (Mirando *et al.*, 1990a). Intrauterine injections of oCSP on Days 11 through 15 also resulted in lower IP hydrolysis after *in vitro* stimulation of endometrial tissues with oxytocin as compared to SP-treated controls (Mirando *et al.*, 1990a, 1990b).

Maintenance of plasma progesterone concentrations noted in pregnant ewes may be important in sustaining the proposed progesterone block to OTr formation (McCracken *et al.*, 1984). Ott *et al.* (1992) utilized an ovariectomized ewe model to indirectly (*i.e.* PGFM response to oxytocin challenge and IP hydrolysis) examine the interaction of oCSP and progesterone on OTr formation. Ovariectomized ewes received intrauterine injections of SP or oCSP from Day 11 through 14 (ewes were ovariectomized on Day 4 of the cycle)

and daily progesterone injections (im) were administered from Day 4 through Day 10 or Day 4 through Day 15. The oxytocin-induced PGFM response was completely blocked in oCSP-treated ewes which received progesterone until Day 15. Endometrial tissue of these ewes was not responsive to oxytocin-induced IP hydrolysis *in vitro*, while there was a doubling of the rate of IP hydrolysis in ewes treated with SP and progesterone. However, oCSP did not block endometrial responsiveness (either PGFM or IP hydrolysis) if progesterone treatment was stopped on Day 10. Interestingly, oxytocin-induced PGFM increases and IP hydrolysis were also blocked by treatment with progesterone alone until Day 15 (*i.e.*, without intrauterine injection of oCSP). This supports McCracken's proposal that progesterone acts to block the increase in OTr formation until the time of luteolysis, but this does not support McCracken's proposed requirement for estrogen.

There is some evidence that ovine conceptuses secrete a factor (not oIFNτ) that directly affects the luteal cells to attenuate $PGF_{2\alpha}$-induced luteolytic effects. Wiltbank *et al.* (1992) found that *in vitro* cultures of separated large and small luteal cells were affected by oCSP. When large cells were cultured with oCSP and $PGF_{2\alpha}$ the normal anti-steroidal effect of $PGF_{2\alpha}$ alone was blocked. There was no effect of oCSP on progesterone secretion by large luteal cells. However, oCSP increased progesterone secretion from small

luteal cells regardless of whether the small luteal cells were stimulated by LH or not. The anti-$PGF_{2\alpha}$ factor in oCSP, while not fully characterized is apparently a protein as the protective action was lost when oCSP was heated. The anti-$PGF_{2\alpha}$ effect was shown not to be oIFNτ when progesterone secretion was not decreased in the presence of $PGF_{2\alpha}$ and oCSP with oIFNτ removed. While prostaglandin-E has been reported to possess luteoprotective properties (Henderson *et al.*, 1977), prostaglandin-E was not considered the anti-$PGF_{2\alpha}$ factor since their dialysis procedure should have removed all prostaglandin-E. Also, there was no increase in progesterone secretion by the large luteal cells as noted when prostaglandin-E is added to large luteal cell cultures (Fitz *et al.*, 1984). The anti-$PGF_{2\alpha}$ protein in oCSP apparently does not competitively inhibit binding of $PGF_{2\alpha}$ to its receptor as there was not a decrease in binding of ^{3}H-$PGF_{2\alpha}$ in the presence of oCSP. Therefore, it was proposed that the inhibitory action of this putative protein factor was at the post-receptor level, possibly at the level of the second messenger system. This study indicated that a factor in oCSP is capable of protecting progesterone production by luteal cells in the presence of $PGF_{2\alpha}$, and may explain why progesterone secretion is not decreased in pregnant ewes with higher basal levels of $PGF_{2\alpha}$ (Ellinwood *et al.*, 1979; Fincher *et al.*, 1986; Zarco *et al.*, 1988a; Vallet *et al.*, 1989a; Burgess *et al.*, 1990) and why

luteolysis in pregnant ewes requires higher doses of exogenous $PGF_{2\alpha}$ (Inskeep *et al.*, 1975; Silvia *et al.*, 1984b; Silvia *et al.*, 1986). However, this factor is not oIFNτ, shown to be the only protein in oCSP to act on endometrial tissues to prevent pulsatile secretion of $PGF_{2\alpha}$, thus maintaining pregnancy (Vallet *et al.*, 1988). A supportive role of this, to-date unknown protein, to oIFNτ in the maintenance of pregnancy should not be ruled out.

Since intrauterine injection of oCSP attenuates oxytocin-induced $PGF_{2\alpha}$ secretion (Fincher *et al.*, 1986; Vallet *et al.*, 1988) and IP hydrolysis (Mirnado *et al.*, 1990b) by endometrium, it has been proposed that OTr formation is blocked in pregnant ewes by conceptus-mediated events (Flint *et al.*, 1994, 1995; Flint, 1995). Vallet and Lamming (1991) reported that intrauterine injection of oCSP blocked oxytocin-induced $PGF_{2\alpha}$ secretion and endometrial OTr formation. This was the first direct measure which confirmed that conceptus secretory products had an effect on OTr formation.

The proposed progesterone block to OTr formation was suggested to be maintained by the conceptus to prevent normal down regulation of the Pr on Days 12 to 14 (McCracken *et al.*, 1984). In the pregnant ewe, Pr concentrations in endometrial tissues does not change from Day 10 to Day 16 (Ott *et al.*, 1993b). However, during this time Pr mRNA actually decreased by 50 percent. Estrogen receptor protein

and mRNA decreased in pregnant ewes from Day 10 to Day 16. Mirando *et al.*, (1993) reported that intrauterine injection of oCSP decreased Er protein and mRNA, as well as OTr in endometrial tissues. These results support an earlier hypothesis proposed by Ott *et al.* (1993) that Pr are stabilized in pregnant ewes and that Er formation is blocked. This in turn prevents the formation of endometrial OTr and, therefore, release of luteolytic pulses of $PGF_{2\alpha}$ in response to oxytocin by endometrium. Progesterone dependence has also been reported for uterine responsiveness to oxytocin (as measured by IP metabolism; Vallet *et al.*, 1989b; Ott *et al.*, 1992). However, as indicated previously, others have shown that continuous exposure of the endometrium to progesterone down-regulates endometrial Pr mRNA and protein abundance in the luminal epithelium, shallow glandular epithelium, and stroma (Wathes and Hamon, 1993; Spencer and Bazer, 1995). The mechanism responsible for this is currently poorly understood, but may involve Pr-mediated decreases in Pr gene transcription (Alexander *et al.*, 1989; Read *et al.*, 1988). Results from studies performed by Spencer *et al.* (1995) have shown that negative regulation of the Pr gene in the endometrial epithelium occurs in both cyclic and pregnant ewes, because Pr mRNA abundance and immunoreactive Pr protein declined in the endometrial luminal epithelium and shallow glandular epithelium after Day 6. Thus, results of this study were

used to modify the hypothesis to indicate that pregnancy does not stabilize or up-regulate Pr gene expression in the endometrium.

Ovine Trophoblast Protein-1

To determine whether ovine conceptuses produce a pregnancy recognition factor, ovine conceptuses were collected on Days 13 through 21 and cultured in the presence of ^{3}H-leucine for 24 h (Godkin *et al.*, 1982). Two-dimensional polyacrylamide gel electrophoresis of the dialyzed medium reveled one major protein product with three isoelectric species. The isoelectric species had pI's of 5.5 to 5.7 and an estimated molecular weight of 17,000 to 20,000. This conceptuses product was initially referred to as protein X (Wilson *et al.*, 1979; Godkin *et al.*, 1982; 1984a) Protein X production could be detected by gel filtration chromatography 2D PAGE between Days 13 and 21 of pregnancy (Wilson *et al.*, 1979; Godkin *et al.*, 1982). *In situ* hybridization studies later showed that oTP-1 mRNA could be detected on Day 12, but full scale production of oTP-1 was not up-regulated until Day 13 (Hansen *et al.*, 1985; Farin *et al.*, 1990). Because this protein was produced transiently by the conceptus during the period of maternal recognition it was proposed to be the pregnancy recognition factor (Godkin *et al.*, 1982). This same protein

had been partially characterized by Martal and coworkers in 1979 and named Trophoblastin.

Godkin *et al.* (1984a) further characterized the actions of oTP-1 in a series of experiments which showed that oTP-1 in Day 16 pregnant sheep was localized within the uterus. Ovine trophoblast protein-1 was associated with the trophectoderm cells of the blastocyst and with the surface and upper glandular epithelium of the uterus. Uterine infusion of ^{125}I-oTP-1 into Day 12 cyclic ewes indicated that oTP-1 was retained in the uterine tissues with little reaching the vasculature draining the uterus. When tissues from the CL and other ovarian structures were examined no oTP-1 was found. This indicated that the action of oTP-1 is local, at the level of the endometrium. Ovine trophoblast protein-1 did not stimulate progesterone production by dispersed luteal cells from Day 12 cycling ewes. However, there was an oTP-1-induced increase in protein production *in vitro* from uterine tissue acquired from ewes on Day 12 of their cycle. In competition assays, oTP-1 did not compete with oPRL in rabbit mammary cell cultures. Ovine trophoblast protein-1 also did not compete with hCG or bLH in sheep luteal cell cultures. As a result of these studies, the authors suggested three possible functions for oTP-1: first; (1) induce the uterus to produce proteins to meet the nutritional requirements of the conceptus until attachment occurs; (2) induce endocrinological changes

within uterine tissues which control the synthesis, release, or sequestration of $PGF_{2\alpha}$; or (3) induce secretion of particular proteins from the endometrium which would act in a luteotropic fashion at the level of the ovary. The second and third functions have been shown to be incorrect (for oTP-1). For this reason oTP-1 is considered to be an antiluteolytic hormone and not a luteotropic hormone.

A protein similar to oTP-1 is produced by the bovine and caprine conceptuses, and termed bTP-1 and cTP-1, respectively. The bTP-1 is the major protein produced by the bovine conceptus during the period of maternal recognition of pregnancy (Day 16 to 24). Bovine trophoblast protein-1, like oTP-1, posses several molecular weight and isoelectric variants ranging from 20 to 26 kDa and pI of 4.5 to 6.5, respectively (Bartol *et al.*, 1985). However, unlike oTP-1, bTP-1 is glycosylated. Caprine trophoblast protein-1 has at least two isoforms with molecular weights of about 17,000 and pIs of 5.2-5.7. The cTP-1 is the major protein produced by the goat conceptus during the time of maternal recognition of pregnancy (Day 17) (Gnatek *et al.*, 1989).

Ovine Trophoblast Protein-1 is an Interferon

The identification of oTP-1 as an IFN came about as result of molecular cloning of cDNA and protein sequencing techniques (Imakawa *et al.*, 1987; Stewart *et al.*, 1987; Imakawa *et al.*, 1989; Charpigny *et al.*, 1988). These

reports identified oTP-1 (which has also been called Trophoblastin, Protein-X, oTP-1, oIFNα_{II}1, oTIFN-omega), as a Type I IFN. Ovine trophoblast protein-1 is reportedly closest in homology to the omega IFNs (originally refereed to as alpha$_{II}$1 IFNs). Trophoblast interferons of different species (bTP-1, cTP-1 and oTP-1) are more closely related to one another than they are to the omega IFNs of their own species (i.e. oTP-1 and other ovine omega IFNs). The trophoblast interferons are apparently functionally related as well, as indicated by extension of the cycle in goats when sheep conceptuses were transferred to the goats uterus, prior to the period of maternal recognition or when roIFNτ was injected into the uterine lumen (see Bazer *et al.*, 1993). In cattle, the transfer of trophoblastic vesicles (Heyman *et al.*, 1984) or intrauterine injection of roIFNτ also results in extension of the cows estrous cycle (see Bazer *et al.*, 1993). It has been proposed, for these reasons, that the nomenclature IFNτ be used to refer to all the trophoblast interferons (ovine, oIFNτ; bovine, bIFNτ; and caprine, cIFNτ) Genes for IFNs are believed to exist in all ruminants of the order Artiodactyla and are believed to have diverged from IFN-omega genes 30 to 65 million years ago (Roberts *et al.*, 1992; Leaman *et al.*, 1992). Several cDNA sequences have been published for oIFNτ since the first reports; however, they all have common characteristics

(Stewart *et al.*, 1989b; Klemann *et al.*, 1990; Charlier *et al.*, 1991).

All IFNτs are 172 amino acids in length. Trophoblast interferons, like other Type I IFNs, are intron-less. They are coded for by a 595 base pair open reading frame which codes for a 195 amino acid preprotein with a 23 amino acid signal sequence that is cleaved to produce the 172 amino acid mature protein. There are two disulfide bridges at highly conserved Cys residues. The first is between the Cys^{1} residue and Cys^{99}. The second is between Cys^{29} and Cys^{139}. This last pair of Cys residues have been identified in all alpha, beta and omega IFNs and appears to be important for biological activity of the molecule (see Roberts *et al.*, 1992). There are several other conserved residues found in all Type I IFNs, including the IFNτs. They are the four Cys just discussed, as well as Leu^{3}, Leu^{30}, Arg^{33}, Phe^{38}, Pro^{39}, Glu^{50}, Glu^{52}, Ser^{73}, Gln^{92}, Leu^{96}, Tyr^{123}, Tyr^{130}, Leu^{131}, Ala^{140}, Trp^{141}, and Val^{144} (Klemann *et al.*, 1990).

Hydrophilicity-hydrophobicity plots of the different isoforms of Type I IFN are very similar in spite of the differences noted in the sequences as a whole. Secondary structures are also very similar. It is believed that there are five α-helices arranged in an anti-parallel manner with connected loop regions (see Roberts *et al.*, 1992). This arrangement is similar to that reported for IFNß, interlukin-1, interlukin-4, growth hormone and granulocyte

macrophage-colony stimulating factor (see Bazer *et al.*, 1993). Interestingly, a recent report indicates that granulocyte macrophage-colony stimulating factor may play a role in modulation of production of oIFNτ (Imakawa *et al.*, 1993)

The oIFNτ molecule is not glycosylated; however, there is a site of potential glycosylation in some of the isoforms at Asn^{78} (Godkin *et al.*, 1982; Anthony *et al.*, 1988) This is in contrast to bIFNτ and some isoforms of cIFNτ which are glycosylated (Helmer *et al.*, 1987; Helmer *et al.*, 1988; Baumbach *et al.*, 1990). Why oIFNτ is not glycosylated and other IFNτs are is not currently known.

Trophoblast IFNs are biologically similar to other Type I IFNs. Ovine IFNτ affords antiviral protection to cells cultured in the presence of virus just as IFNα (Pontzer *et al.*, 1988). Ovine IFNτ decreases proliferation of several cell lines as does IFNα (Pontzer *et al.*, 1991). Incorporation of tritiated thymidine into lymphocytes following mitogen exposure is blocked by both oIFNτ and IFNα (Newton *et al.*, 1989). Finally, oIFNτ up-regulates 2,5-oligoadenylate synthetase in endometrial tissues (Mirando *et al.*, 1991). The major difference between the IFNτs and other Type I IFNs is their apparent lack of cytotoxicity. Even in large concentrations, the IFNτs exert little or no cytotoxic effects. This lack of cytotoxicity has generated

considerable interest in IFNτ for use as a therapeutic drug (see Bazer, 1991).

For future experiments in the area of maternal recognition of pregnancy, and as a therapeutic drug, IFNτ must be produced in larger quantities than those obtained from 30 hour cultures of a Day 16 conceptus (Godkin *et al.*, 1982). For this reason, recombinant forms of oIFNτ have been developed using synthetic oligonucleotides and cDNAs (Ott *et al.*, 1991 and Martal *et al.*, 1990, respectively). The roIFNτ produced by Ott *et al.* (1991) was derived from a synthetic gene which was edited to include 17 unique restriction sites not found in the natural oIFNτ sequence (Imakawa *et al.*, 1989). While roIFNτ was designed for expression in *E. coli*, yeast were used to overproduce the product. The use of yeast has allowed large amounts of roIFNτ to be produced (Ott *et al.*, 1991; Van Heeke *et al.*, 1996). The restriction sites will allow for the easier production of mutants to investigate structure/function of oIFNτ domains in the future. Biological activities for roIFNτ and oIFNτ have been shown to be identical (Ott *et al.* 1991).

Maternal Recognition Effects of Ovine Interferon *Tau* and Type I Interferons

While it had been shown that the maternal pregnancy recognition factor produced by the ovine conceptus was contained within the milieu of oCSPs, and that oTP-1 (now

referred to as oIFNτ) was the major protein produced by the conceptus during the pregnancy recognition period (Godkin *et al.*, 1984b), studies with purified oIFNτ were required to demonstrate that oIFNτ is the maternal pregnancy recognition factor.

The ability of partially purified oIFNτ to extend the interestrous interval was initially demonstrated by Godkin *et al.* (1984b). The purified oIFNτ was obtained by pooling culture media from a large number of conceptus cultures and passing the media over DEAE cellulose ion-exchange and S-200 Sephacryl columns. The resultant purified product was injected into the uterine lumen via catheters surgically placed into the tip of each uterine horn on Days 12 through 21. Plasma progesterone was used to determine CL life-span. A decrease in plasma progesterone concentrations to below 1 ng/ml was used to indicate luteal regression. Progesterone levels in ewes treated with partially purified oIFNτ remained elevated 4 days longer than controls. While this was significant it was not nearly as long as the cycle extension noted for ewes (from this same report) which received intrauterine injections of oCSP. The differences in the extension of cycle between ewes which received oCSP and those that received oIFNτ could have been due to several factors. The purified oIFNτ may have been degraded by uterine protease or the estimate of the amount used per infusion, calculated from conceptus production in culture

during a 24 hour period, may have been too low to elicit the same response as oCSP. The other major question was whether the difference between the two treatments could be due to some other factor which acted in concert with the oIFNτ in the total oCSP to cause the longer cycle extension. To answer these questions, Vallet *et al.* (1988) prepared highly purified oIFNτ. In addition, the oCSP remaining after oIFNτ had been removed using an anti-oIFNτ column several times to remove all traces of oIFNτ, was used to treat ewes. Ewes were fitted with uterine catheters and injected (intrauterine) with either SP, oCSP, oIFNτ, or oCSP minus oIFNτ. There was no difference in the cycle length (19 days each; determined by fall in plasma progesterone) between ewes treated with SP and oCSP with oIFNτ removed. From these findings, it is apparent that proteins other than oIFNτ in oCSP are not involved in maternal recognition of pregnancy. Further, there was no difference in the intraestrous interval between the ewes which received oCSP or highly purified oIFNτ (27 and 25 days, respectively). These results indicate that there is not a synergistic effect between other conceptus products and oIFNτ in maternal recognition of pregnancy.

Intrauterine injections of recombinant forms of oIFNτ also increase the interestrous interval in ewes. Martal *et al.* (1990) reported an extension of up to 64 days (confirmed by marked CL at slaughter) when large amounts of roIFNτ were

administered (intrauterine; 340 μg/day). There was also an extension of the cycle in ewes which received levels of roIFNτ comparable to that produced by Day 16 conceptus in culture (170 μg/day), as shown in more recent studies in which roIFNτ was administered from Day 11.5 through Day 16 and resulted in an extension of the interestrous interval to that of about 31 days (OTT *et al.*, 1993a). Recombinant forms of other Type I IFNs (rbIFNα) also extend the interestrous interval; however, larger doses were required (2 mg/day) (Stewart *et al.*, 1989a; Parkinson *et al.*, 1992). It is interesting to note that intramuscular injections of rbIFNα increased the lambing rate in treated ewes (Schalue *et al.*, 1989, 1991; Nephew *et al.*, 1990; Martinod *et al.*, 1991). Although, it is not known how rbIFNα aided in pregnancy recognition it was proposed that it supplemented the activity of endogenous oIFNτ to ensure recognition of pregnancy in ewes in which conceptuses may have been retarded in growth, or for some reason producing suboptimal levels of oIFNτ.

Effects on prostaglandin Synthesis

Intrauterine injections of oIFNτ affect endometrial prostaglandin responsiveness to oxytocin and estrogen in a manner similar to that following intrauterine injections of oCSP in studies by Fincher *et al.* (1986). Vallet *et al.* (1988) reported that a challenge with estradiol on Day 14

resulted in a subsequent rise of plasma PGFM concentrations in ewes treated with intrauterine injections of SP as controls while there was no such rise in ewes which received oIFNτ. These same ewes, when challenged with oxytocin on Day 15, responded with a lower PGFM response when treated with oIFNτ. There was no effect of treatment on prostaglandin-E production. This indicates that the prostaglandin production is not shunted from production of $PGF_{2\alpha}$ in cyclic ewes, to production of prostaglandin-E in pregnant ewes, to prevent luteolysis as proposed by McCracken *et al.* (1984).

Attenuation of the $PGF_{2\alpha}$ response to exogenous oxytocin in pregnant ewes is acquired over a period of several days. Endometrium collected from ewes on Day 15, after treatment with oIFNτ or SP by intrauterine injection on Days 12 through 14 (Vallet *et al.*, 1989a) was perifused with buffer plus oxytocin and the medium assayed for $PGF_{2\alpha}$ content. There was a higher $PGF_{2\alpha}$ response to oxytocin for SP-treated than for oIFNτ-treated ewes. This supported results from a previous *in vivo* study (Fincher *et al.*, 1986). However, when Day 15 endometrium was obtained from ewes not treated with oIFNτ *in vivo*, the *in vitro* $PGF_{2\alpha}$ response was higher for endometrium treated *in vivo* with oIFNτ. This is exactly opposite from the effects reported with long term treatment with oIFNτ. These results imply that oIFNτ does not act directly to competitively interfere with binding of oxytocin

to its receptor or by other means to block the $PGF_{2\alpha}$ response. Rather, oIFNτ, prevents development of endometrial sensitivity to oxytocin-induced $PGF_{2\alpha}$ secretory response. It has been shown that Type I interferons increase arachidonic acid metabolism. If oIFNτ has the same affect on uterine tissues the increase in $PGF_{2\alpha}$ secretion reported in the short term perifusion experiments may be attributed to an increase in arachidonic acid metabolism within the tissues. However, several reports in the literature indicate that, in uterine tissues, this may not occur (Salamonsen *et al.*, 1988) and that arachidonic acid mobilized by IFNα is shunted away from the cyclooxygenase pathway (Hannigan and Williams, 1991).

As mentioned previously, IFN produced by the bovine conceptus is very similar to that of oIFNτ. Bovine IFNα and other Type I IFNs, like oIFNτ, extend CL life-span when administered at the time of maternal recognition in the cow (Thatcher *et al.*, 1989; Helmer *et al.*, 1989a: Plante *et al.*, 1991). However, unlike the ewe, basal $PGF_{2\alpha}$ is lower in both pregnant cows, and cows which receive IFN treatment, than in cyclic cows. It is not known why basal levels of $PGF_{2\alpha}$ are lower in bIFNτ-treated cows compared to controls, while there is no difference in basal levels of $PGF_{2\alpha}$ noted in oIFNτ-treated and control ewes. It may be that the cow blocks luteolysis by inhibition of $PGF_{2\alpha}$ synthesis early in the $PGF_{2\alpha}$/oxytocin feedback loop to prevent a rise in OTr.

There is evidence for this mode of action of bIFNτ in cows (Shemesh *et al.*, 1981; Basu and Kindahl, 1987; Gross *et al.*, 1988a; Helmer *et al.*, 1989b) The action of a prostaglandin inhibitor in sheep has not been well studied, but endogenous prostaglandin inhibitors have been detected in endometrium (Basu, 1989) and in allantoic fluid (Harper and Thornburn, 1984; Rice *et al.*, 1987) of sheep. It is not known, however, if an inhibitor of $PGF_{2\alpha}$ synthesis plays a role in maternal recognition pregnancy in ewes. In the cow, bIFNτ may induce synthesis of a prostaglandin inhibitor (Danet-Desnoyers *et al.*, 1993). Interferons affect arachidonic acid metabolism. Also, inhibitors of the cyclooxygenase or lipoxygenase pathways of arachidonic metabolism increase activities of the transduction signal for IFNα (Hannigan and Williams, 1991). Type I IFNs have been reported to attenuate prostaglandin secretion. Interferon-α was reported to attenuate prostaglandin production in human cells (Dore-Duffy *et al.*, 1983; Browning and Ribolini, 1987) The prostaglandin affected in this study was prostaglandin-E which is produced via the same pathway (cyclooxygenase) as $PGF_{2\alpha}$. Human IFNα incubated with endometrial cells from ovariectomized ewes, maintained on a steroid regime that mimics that of a normal estrous cycle, caused a decrease in $PGF_{2\alpha}$ secretion (Salamonsen *et al.*, 1988; Salamonsen *et al.*, 1989). Vallet *et al.* (1991) reported that intrauterine injection of rbIFNα on Days 12 through 14 was as effective

in blocking oxytocin-induced $PGF_{2\alpha}$ secretion from the uterus as was oCSP; however, this was believed to be through progesterone attenuation of the OTr.

The Ovine Interferon *Tau* Receptor and Signal Transduction

Ovine IFNτ, like other Type I IFNs (alpha, beta, and omega) bind to a high affinity (Godkin *et al.*, 1984a;) Type I IFN receptor (Stewert *et al.*, 1987). Type I IFN receptors are distributed throughout endometrial tissues of the ewe and their expression may be influenced by ovarian steroids (Knickerbocker and Niswender, 1989). Type I receptors are also present in other tissues of the body (Knickerbocker and Niswender, 1989). Pontzer *et al.* (1990) reported that high concentrations of the NT of oIFNτ attenuated antiviral effects of oIFNτ, but not IFNα, in cell co-cultures. Conversely, a synthetic peptide corresponding to amino acids 139-172 (CT) blocked antiviral effects of both oIFNτ and IFNα in cell co-cultures. The authors concluded that NT of oIFNτ binds to a unique domain in the Type I IFN receptor while the CT binds to a domain common to Type I IFNs. This may explain the unique actions of oIFNτ.

Ovine IFNτ-induced hormone action is initiated by the transduction of signal via activation of the JAK/STAT system (see Willians, 1991a) and is believed to act in the same manner as other Type I interferons (see Interferon Receptor/Signal Transduction section).

Ovine IFNτ and other IFNτ's increase endometrial protein production dramatically (Gross *et al.*, 1988b; Sharif *et al.*, 1989; Ashworth and Bazer, 1989). Included in these is the enzyme 2',5'-oligoadenylate synthetase (Mirando *et al.*, 1991; Short *et al.*, 1991). Estrogen receptors are increased in endometrial adenocarcinoma cells by IFNα_{2b} and in human breast cancer tissue and human endometrium. In rabbit endometrium Er expression is increased by IFNα. Progesterone receptors may be increased by IFNα_{2b} in endometrial adenocarcinoma and by IFNα in AE-7 endometrial cancer cells (see Bazer *et al.*, 1993). Full length Er and Pr genes for ruminants have not been cloned to determine the presence of an interferon stimulated response element in the 3'or 5' flanking region; however, analysis of partial clones of genomic DNA from human and rabbit Er and Pr indicate their presence (see Bazer *et al.*, 1993). If an interferon stimulated response element(s) is present in the Er and Pr genes they may allow oIFNτ to negatively regulate expression of Er and OTr within the endometrium during early pregnancy (Mirando *et al.*, 1993; Ott *et al.*, 1993b) to allow for establishment of pregnancy.

CHAPTER 3

THE EFFECTS OF RECOMBINANT OVINE INTERFERON *TAU* AND SYNTHETIC PEPTIDES, CORRESPONDING TO PORTIONS OF RECOMBINANT OVINE INTERFERON *TAU*, ON OXYTOCIN-STIMULATED ENDOMETRIAL INOSITOL PHOSPHATE METABOLISM AND ENDOMETRIAL OXYTOCIN RECEPTOR CONCENTRATION.

Introduction

The establishment of pregnancy in ewes requires that the conceptus, by Day 12 of pregnancy (Moor and Rowson, 1966a), activate a mechanism(s) (oIFNτ; Godkin *et al.*, 1982; Vallet *et al.*, 1988) to prevent luteolysis. Luteolysis in ruminants is initiated by pulsatile secretion of $PGF_{2\alpha}$ by endometrial tissues (McCracken *et al.*, 1981; Hooper *et al.*, 1986). Pulsatile secretion of uterine $PGF_{2\alpha}$ is thought to be responsible for luteolysis due to the fact that continuous infusion of $PGF_{2\alpha}$, or immunization against $PGF_{2\alpha}$, results in extension of the cycle (Scaramuzzi and Baird, 1976; Fairclough *et al.*, 1981). During luteal regression, $PGF_{2\alpha}$ is secreted from the endometrium in a series of five to eight, high amplitude, short duration episodes (Thornburn *et al.*, 1973; Barcikowski *et al.*, 1974; Flint and Sheldrick, 1983; Zarco *et al.*, 1988b) with 6 to 8 h between each episode. McCracken *et al.* (1984) have shown that the CL must be exposed to approximately 5 pulses of $PGF_{2\alpha}$ over a 25

h period to undergo complete luteolysis. The pulsatile secretion of $PGF_{2\alpha}$ may be initiated by the secretion of oxytocin from the posterior pituitary and is escalated by oxytocin secreted by the CL (Flint *et al.*, 1990). Oxytocin from the CL and $PGF_{2\alpha}$ from the uterus act together in a positive feedback loop to generate the luteolytic pulses required for luteolysis (Flint and Sheldrick., 1986; Hooper *et al.*, 1986; see Silvia *et al.*, 1991 ;McCracken *et al.*, 1991). However, the ability of the endometrium to secrete $PGF_{2\alpha}$ in response to oxytocin does not develop until Day 13 to 14 of the cycle (Roberts *et al.*, 1976; Roberts and McCracken, 1976; Fairclough *et al.*, 1984; Silvia *et al.*, 1991) when OTr concentrations increase (Sheldrick and Flint, 1985). It is the coupling of oxytocin to specific endometrial OTr sites that stimulates $PGF_{2\alpha}$ synthesis, through activation of the inositol phosphate/diacylglycerol signal transduction pathway (Flint *et al.*, 1986; Silvia and Homanics, 1988).

During early pregnancy, pulsatile secretion of $PGF_{2\alpha}$ secretion by the uterus is attenuated or absent (Thornburn *et al.*, 1973; Barcikowski *et al.*, 1974; Moore and Watkins, 1982; Hooper *et al.*, 1987; Zarco *et al.*, 1988a) with a disruption of the oxytocin/$PGF_{2\alpha}$ positive feedback loop. The attenuation of the loop in the pregnant ewe is primarily due to the absence of expression of endometrial OTr and Er

(McCracken *et al.*, 1984; Sheldrick and Flint, 1985, Spencer *et al.*, 1995b).

Ovine IFNτ is the antiluteolytic protein secreted by the conceptus (Godkin *et al.*, 1992; Vallet *et al.*, 1988; see Bazer *et al.*, 1991; Godkin *et al.*, 1984a; 1984b; Bazer *et al.*, 1995). Endometrial IP metabolism (Mirando *et al.*, 1990 a,b; Ott *et al.*, 1992), $PGF_{2\alpha}$ secretion (Vallet *et al.*, 1988; Mirando *et al.*, 1990a; Ott *et al.*, 1992) as well as endometrial OTr and Er expression (Vallet and Lamming, 1991; Mirando *et al.*, 1993; Spencer *et al.*, 1996) are inhibited by intrauterine injection of oIFNτ. Intrauterine injection of roIFNτ is as effective as oIFNτ purified from conceptus culture medium in blocking luteolysis (Ott, 1992; Ott *et al.*, 1993a). It is believed that oIFNτ acts through its Type I IFN receptor, and activation of its transduction signal to prevent endometrial expression of Er and OTr. The Pr and progesterone are considered permissive to antiluteolytic effects of oIFNτ, but the mechanism is not known (Spencer 1995).

Several questions have been raised as to whether or not the oxytocin-induced IP metabolism, previously reported from our laboratory, was due to stimulation through the OTr, or could it be through the AVP receptor. Experiment 1 was designed to answer this question, by determining if oxytocin-induced IP metabolism within endometrial tissues is mediated through the OTr or the AVP receptor. This was

accomplished by means of an IP metabolism assay that examined all possible combinations of OT, and AVP stimulation with all possible combinations of the receptor antagonist for the OTr and AVP receptor.

Other primary objectives of these experiments (as they relate to IP metabolism and endometrial OTr concentration), were to determine what effect treatment of cyclic ewes, with various synthetically produced peptides corresponding to overlapping segments of oIFNτ, had on oxytocin-induced endometrial IP metabolism, and on endometrial OTr concentration. The NT and CT peptides were first examined (Experiment 2) due to the fact that they were the most extensively examined of the peptides (Pontzer *et al.*, 1990, 1994). It has been proposed that the NT possesses the properties which makes oIFNτ different from other IFNαs, and that CT is the portion common to the IFNαs. Experiment 3 examined the effect of the remaining peptides (2-5) on oxytocin-induced IP metabolism and endometrial OTr concentration. Experiments 4, also examined the effect of NT treatment on endometrial OTr concentration, but this experiment was specifically designed to determine what effect oxytocin challenge (*in vivo*) on Days 13 and 15 would have with regards to the main treatment of NT or roIFNτ. The final experiment, Experiment 5, was designed to determine what effect NT has on endometrial OTr concentration through Day 18, which is two days longer than

oIFNτ had been examined. This was to determine if NT has the ability to hold OTr concentration at a low level for an extended period of time.

Materials and Methods

Animals

Ewes of primarily Rambouillet breeding were checked daily at 07:30 am for 20 min with vasectomized males of St. Croix or mixed Rambouillet breeding. Ewes which had previously exhibited at least two normal estrous cycles (16 to 17 days in length) were assigned to experimental groups. Ewes for experiments 1, 2, and 3 were housed at the Sheep Research Facility, University of Florida, Gainesville. Ewes for experiment 4 and 5 were housed at the Sheep Research Center, Texas A&M University, College Station.

Protein And Peptide Preparation

Ovine conceptus secretory protein preparation

Ovine conceptus secretory proteins were prepared as previously reported by our laboratory (Vallet *et al.*, 1988 and Mirando *et al.*, 1990b). Briefly, ovine conceptuses were collected at laparotomy on Day 16 of pregnancy by flushing the uterus with 20 ml minimum essential medium (Earl's salts; Gibco/Life Technologies, Grand Island, New York). The conceptuses were cultured for 30 h in minimum essential medium as reported by Godkin *et al.* (1982). The resultant

oCSP-conditioned medium was collected, pooled and stored at -20°C until used.

Medium containing oCSP was thawed, pooled and dialyzed (3500 Mr cutoff) at 4°C against 4 liters of 0.9% NaCl (w/v), changed three times (4L each change). Ovine conceptus secretory proteins were concentrated to one-tenth the original volume using an Amicon ultrafilter (500 Mr cutoff; Amicon Co., Danvers, MA). The concentration of oIFNτ in oCSP was determined by RIA (Vallet *et al.*, 1988). Ovine conceptus secretory proteins were diluted with in 0.9% NaCl (w/v) to an oIFNτ concentration of 25μg/ml oIFNτ. The concentration of total protein in oCSP was determined by the method of Lowry *et al.* (1951). The oCSP was diluted with SP in 0.9% NaCl (w/v) to a total protein concentration of 0.75 mg/ml, and stored at -20°C in 2 ml aliquots until just prior to use when they were thawed under running water.

Recombinant interferon *tau* preparation

Recombinant oIFNτ, provided by Dr. Troy Ott, was produced as described by Ott *et al.* (1991). Antiviral units of roIFNτ were determined by antiviral assay using Madin Darby bovine kidney cells challenged with vesicular stomatitis virus (Pontzer *et al.*, 1988). Protein concentration of roIFNτ was determined by protein assay (Lowry *et al.*, 1951). Recombinant oIFNτ was diluted to 25 μg/ml for Experiment 3 and 50 μg/ml for Experiments 4 and 5. The total protein concentration was brought up to 0.75 mg/ml

by the addition of SP in 0.9% NaCl (w/v). Aliquots of 2 ml each were stored in glass scintillation vials at -20°C until just prior to use at which time they were thawed under running water.

Serum protein preparation

Blood was collected from the jugular vein of a pregnant ewe on Day 16 (07:00 am) and allowed to clot 1 h at room temperature and then overnight at 20°C. Serum was collected and dialyzed (3500 Mr cutoff) at 4°C against 4 L 0.9% NaCl (w/v), with three changes (4 L each change). Protein concentration was determined (Lowry *et al.*, 1951) and diluted to a protein concentration of 0.75 mg/ml with 0.9% NaCl (w/v) and stored in glass scintillation vials at -20°C in 2 ml aliquots until just prior to use when they were thawed under running water.

Synthetic peptide production

Synthetic peptides corresponding to the amino and carboxyl-terminus of oIFNτ were produce by Dr. Carol Pontzer in Dr. Howard M. Johnson's laboratory as described by Pontzer *et al.* (1990). Briefly, peptides were synthesized on a Biosearch 9500AT automated peptide synthesizer using fluorenylemthyloxycarbonyl chemistry. Peptides were cleaved from resins using trifluroacetic acid/ethanedithiol/thioanisole/anisole. Cleaved peptides were extracted in diethyl ether and ethyl acetate, dissolved in water and lyophilized. Reverse phase HPLC was used to

determine purity of the peptides. Peptides produced were: to the NT (aa 1-37); CT (aa 139-172): as well as four overlapping internal peptides: Pep 2 (aa 34-64); Pep 3 (aa 62-92); Pep 4 (aa 90-122); and Pep 5 (aa 119-150). All peptides were reconstituted in 0.9% NaCl (w/v) to 0.5 mg/ml. This concentration had previously been shown to block oIFNτ-induced antiviral activity (Pontzer *et al.*, 1990). Serum proteins were added to bring total protein concentration to 0.75 mg/ml in a 2 ml volume. Aliquots of 2 ml were stored in glass scintillation vials at -20°C until just prior to use when they were thawed under water.

Experimental Design

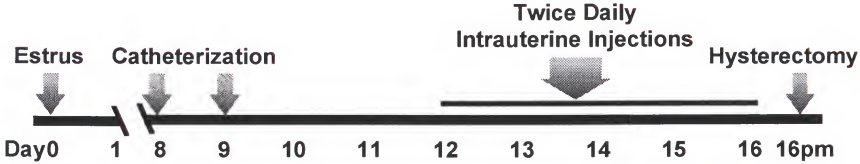
Experiment 1

To determine if oxytocin-induced IP metabolism within endometrial tissues is mediated through the OTr or the AVP receptor inositol phosphate metabolism was examined in endometrium from three ewes after *in vitro* stimulation of oxytocin or AVP. Ewes received no *in vivo* treatment. On Day 16 of their estrous cycle ewes, were anesthetized with halothane and ovariectomized-hysterectomized by mid-ventral laparotomy. Caruncular tissue was collected from the entire uterus into 100 mm petri dishes, maintained on ice and minced into fine pieces. Tissue (0.999 gm) was transferred to 16 separate 20 ml glass scintillation vials and placed on ice until the oxytocin/AVP IP assay was begun (~ 10 min).

Experiment 2

Twenty-four ewes were randomly assigned in a 2 X 3 factorial arrangement to receive SP, SP+NT, SP+CT, oCSP, oCSP+NT or oCSP+CT (n=4/treatment; Fig. 3.1). On Day 6, ewes were anesthetized with halothane and the uterus exteriorized by laparotomy. The number and location of CL were recorded, and a catheter (8' in length; V6 tubing, Bolab, Lake Havasu City, Az) placed into each uterine horn (~ 2 cm) via the oviduct at the utero-tubal junction (Vallet *et al.*, 1988). Catheters were secured to the oviduct at the utero-tubal junction, and the uterine body at the external bifurcation, with suture on either side of a set of cuffs (~2 cm and 30 cm from one end; V10 Tubing, Bolab, Lake Havasu City, AZ) fused to the catheter with a drop of cyclohexanone (Fisher). Catheters were exteriorized through an incision in the flank, and attached to the skin by suturing cloth tape, which was wrapped around the tubing, to the skin. The catheters were flushed with sterile 0.9% saline to determine that there were no blockages and the ends sealed to prevent air from entering the tubing. The external portion of the tubing was stored, wrapped in betadine soaked 4 X 4 gauze, and placed in a pouch attached by suture to the skin at the point of exit (Vallet *et al.*, 1988). Ewes were allowed to recover for 24 h. They were then returned to their respective group pens until where they were placed into individual pens located within group

Figure 3.1: Experimental design for Experiment 2.



Treatments:

SP 1.5 mg/ uterine horn 2X daily
SP + 0.5 mg NT/ uterine horn 2X daily (aa 1-37)
SP + 0.5 mg CT/ uterine horn 2X daily (aa 139-172)

oCSP (25 μg oIFNτ) / uterine horn 2X daily
oCSP + 0.5 mg NT / uterine horn 2X daily
oCSP + 0.5 mg CT / uterine horn 2X daily

Total protein/intrauterine injection = 1.5 mg (Balanced with SP)
Total volume/intrauterine injection including saline flush = 3.1 ml
Tissues collected for IP, OTr (filter method), and Er mRNA

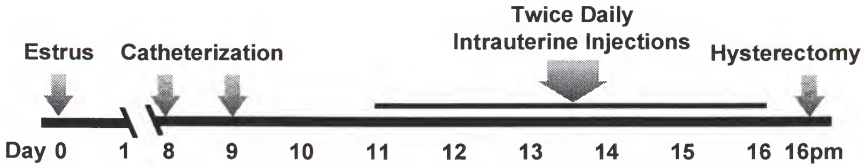
pens until Day 11. This arrangement limited movement of the ewes during the treatment period while allowing them constant contact with the other ewes in their respective pens. Hay and water were available *ad libitum* and about 120 gm concentrate feed was provided each morning. Hay, water and food were withheld 12 h prior to surgery. On Day 12, treatments began and continued through the morning of Day 16. Each ewe received, per uterine horn, twice daily injections (06:00 and 18:00 h, respectively) of one of the following, according to group: 1.5 mg SP; 0.5 mg NT plus 1.0 mg SP; 0.5 mg CT plus 1.0 mg SP; 0.75 mg oCSP (containing 25 μg oIFNτ by RIA) plus 0.75 mg SP; 0.75 mg oCSP plus 0.5 mg NT; or 0.75 mg oCSP plus 0.5 mg CT. All injections were balanced to a total protein concentration of 1.5 mg with SP and were adjusted to 2 ml in volume with 0.9% NaCl (w/v). Each injection also contained 50 mg ampicillin (Polyflex; Aveco Co. Inc., Fort Dodge, IA) in 0.1 ml 0.9% NaCl (w/v) which was mixed with the thawed treatment sample just prior to injection. Each catheter was flushed with 1 ml 0.9% NaCl (w/v) after injection, resulting in a total infused volume of 3.1 ml. The ends of the each catheter was resealed without introducing air and the catheters returned to the pouch in fresh 4 X 4 gauze sponge soaked in betadine. On Day 15, all food and water was removed from the ewes and on the morning of Day 16, after the last intrauterine injection, the ewes were ovariectomized-hysterectomized.

Endometrial tissue (~1.2 gm; primarily of caruncular origin) from the uterine horn ipsilateral to the CL was collected into ice-cold KRB for determination of IP metabolism. The remainder of the endometrium was collected into bags, snap frozen in liquid nitrogen, and stored at -80°C for use in oxytocin receptor assays (filter method) and Er mRNA analysis (Chapter 5).

Experiment 3

Twenty-eight ewes were randomly assigned to one of seven treatment groups to receive SP, roIFNτ, NT, Pep 2, Pep 3, Pep 4, or Pep 5 (n=4/treatment; refer to the section on synthetic peptide preparation for amino acid determination of each peptide; Fig. 3.2). On Day 8 or Day 9 of the estrous cycle ewes were anesthetized and the uterus exteriorized by laparotomy, the number and location of CL noted and catheters placed as described in Experiment 2. Ewes were housed as described in Experiment 2 with the exception that individual crates were located in an adjoining pen in sight of ewes in their respective group pens. Intrauterine injections were administered as in Experiment 2 except that they began on Day 11. Treatments, per horn, consisted of 1.5 mg SP, 0.25 μg roIFNτ, 0.5 mg NT, 0.5 mg Pep 2, 0.5 mg Pep 3, 0.5 mg Pep 4, or 0.5mg Pep 5. The total protein concentration per treatment was brought to 1.5 mg with SP and the total volume adjusted to 2 ml with 0.9% NaCl (w/v). Each injection, also contained 50 mg

Figure 3.2: Experimental design for Experiment 3.



Treatments:

roIFNτ 100 μg/ ewe/ day
25 μg / uterine horn 2X daily

Pep 2 mg/ ewe/ day
500 μg/ uterine horn 2X daily

- NT (aa 1-37)
- Pep 2 (aa 34-64)
- Pep 3 (aa 62-92)
- Pep 4 (aa 90-122)
- Pep 5 (aa 119-150)

SP 6 mg/ day
1.5 mg/ uterine horn 2X daily

ampicillin in 0.1 ml 0.9% NaCl (w/v) which was added with the sample just prior to injection. Each catheter was flushed with 1 ml of 0.9% NaCl (w/v) after injection, to clear the catheter, so the volume injected was 3.1 ml.

On Day 15 all food and water was removed from the ewes and on the morning of Day 16, after the last injection, the ewes were ovariectomized-hysterectomized. Endometrial tissue (~1.2 gm; primarily of caruncular origin) from the uterine horn ipsilateral to the CL was collected into ice-cold KRB for determination of IP metabolism. The remainder of the endometrium was placed in plastic bags, snap frozen in liquid nitrogen, and stored at -80°C for use in oxytocin receptor assays (filter method) and Er mRNA analysis (Chapter 5).

Experiment 4

Twelve ewes were randomly assigned to receive intrauterine injection of either SP, roIFNτ or NT (n=4/treatment; Fig. 3.3 and 3.4). On Day 8 or 9 of the estrous cycle ewes were anesthetized and the uterus exteriorized by laparotomy and catheters placed as described in Experiment 2. Ewes were allowed to recover for 24 h and then returned to a pen, separate from, but in sight of, ewes in their respective group pen until Day 10, when they were placed into individual pens located within sight of ewes in their group pens. Hay and water were available *ad libitum*. On Day 11 treatments began, and continued twice daily (06:00

Figure 3.3: Overall experimental design for Experiment 4.

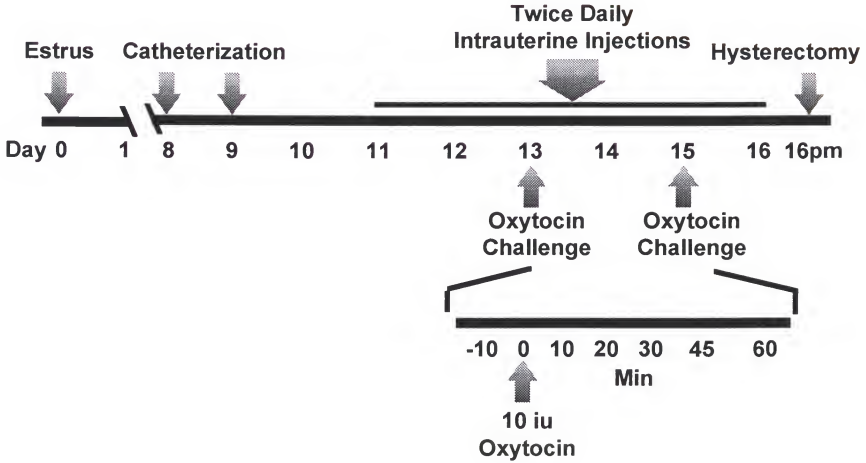
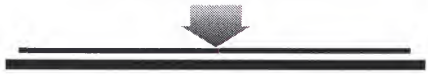

Twice Daily
Intrauterine Injections
Estrus
Catheterization
Hysterectomy
Day 0
1
8
9
10
11
12
13
14
15
16
16pm
Oxytocin
Challenge
Oxytocin
Challenge
-10
0
10
20
30
45
60
Min
10 iu
Oxytocin

Figure 3.4: Detailed design of treatment period for Experiment 4.

Twice Daily
Intrauterine Injections



Total protein / injection = 1.5 mg (balanced with SP)

Total volume / injection including saline flush = 3.1 ml

Tissues collected for PGFM OTr (peg method), Er mRNA, Er and Pr protein

Treatments:

roIFNτ 200 μ g/ ewe/ day
50 μg / uterine horn 2X daily

NT 2 mg/ ewe/ day
500 μg/ uterine horn 2X daily

SP 6 mg/ day
1.5 mg/ uterine horn 2X daily

and 18:00 h) through the morning of Day 16. Treatments, per horn, consisted of 1.5 mg SP, 0.50 μg roIFNτ or 0.5 mg NT. Total protein concentration per treatment was balanced to 1.5 mg with SP and the total volume adjusted to 2 ml with 0.9% NaCl (w/v). Each injection also contained 50 mg ampicillin in 0.1 ml 0.9% NaCl (w/v) which was mixed with the thawed sample just prior to injection. Each catheter was cleared by flushing with 1 ml of 0.9% NaCl after each injection, resulting in a total injected volume of 3.1 ml.

All ewes were challenged with an injection of oxytocin (10 iu) into the jugular vein on Day 14. Jugular blood samples were collected into heparinized tubes, 10 min prior to oxytocin injection at time 0 and at 10, 20, 30, 45, and 60 min post-oxytocin injection. Samples were maintained on ice until they were centrifuged (15 min at 1800 x g and 4°C), plasma transferred to plastic scintillation vials for storage at -20°C until used in PGFM assays (Chapter 4).

On Day 15 all food and water was removed from the ewes and on the morning of Day 16, after the last injection, the ewes were ovariectomized-hysterectomized. Endometrium was collected into plastic bags, snap frozen in liquid nitrogen, and stored at -80°C for use in oxytocin receptor assays (PEG method), as well as analyzed to determine Er mRNA, Er and Pr protein concentrations (Chapter 5).

Experiment 5

Twenty-four ewes were randomly assigned to a 2 x 3 factorial design to receive intrauterine injections of either SP or NT and be hysterectomized on either Day 16, 17 or 18 (n=4/treatment*day; Fig. 3.5). On Day 8 or 9 of the estrous cycle ewes were anesthetized and the uterus exteriorized by laparotomy and catheters placed in the uterine lumen as described in Experiment 2. Ewes were housed as described in Experiment 4. Treatments began the morning of Day 11, and continued through the morning of Day 16. Treatments, per horn, consisted of 1.5 mg SP or 0.5 mg NT. The total protein concentration per treatment was balanced to 1.5 mg with SP and the total volume adjusted to 2 ml with 0.9% NaCl (w/v). Each injection, also contained 50 mg of ampicillin in 0.1 ml 0.9% NaCl (w/v) which was mixed with the thawed sample just prior to injection. Each catheter was cleared by flushing with 1 ml of 0.9% NaCl (w/v) after injection, resulting in a total infused volume of 3.1 ml.

On the morning prior to surgery all food and water was removed from the ewes. Four ewes from each treatment group were ovariectomized-hysterectomized on each of the days examined (Day 16, Day 17 and Day 18). Endometrium was collected into plastic bags, snap frozen in liquid nitrogen, and stored at -80°C for use in oxytocin receptor assays (PEG

method), and analyzed for Er mRNA, Er and Pr protein concentration (Chapter 5).

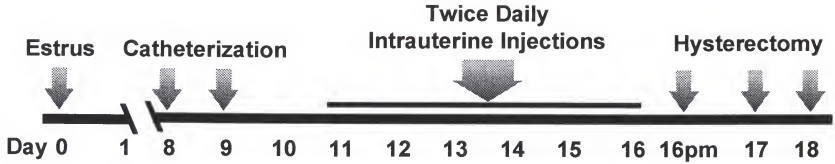
Inositol Phosphate Metabolism

Arginine vasopressin/OT-induced IP metabolism

To determine if changes in IP metabolism were induced within endometrial tissues through AVP receptor-mediated processes, the following assay was modified from that reported by Flint *et al.* (1986) and by Vallet and Bazer (1989).

Caruncular tissue from the ipsilateral uterine horn was collected (Experiment 1) into ice-cold KRB containing 10 mM glucose and 10 μM myo-inositol, minced into approximately 5 mg pieces (Fig. 3.6) Tissue pieces were weighed and 0.1 gm transferred into each of 16 separate glass scintillation vials (see Table 1 for treatments) and washed with 1 ml ice-cold KRB. Tissue incubations were carried out under an atmosphere of 95% O_2, and 5% CO_2; at 37°C in a shaking water bath. Vials were re-gassed and returned to the water bath after each change of buffer until the assay was terminated and vials were placed on ice. Initially, tissues were incubated for 2 h in 1 ml KRB plus 10 μCi myo-[2-^{3}H]inositol (specific activity 18.9 Ci/mmol; Amersham, Arlington Heights, IL). After incubation, KRB was removed and fresh KRB without labeled inositol was added, samples were

Figure 3.5: Experimental design for Experiment 5.



Treatments:

NT 2 mg/ ewe/ day
500 μg/ uterine horn 2X daily

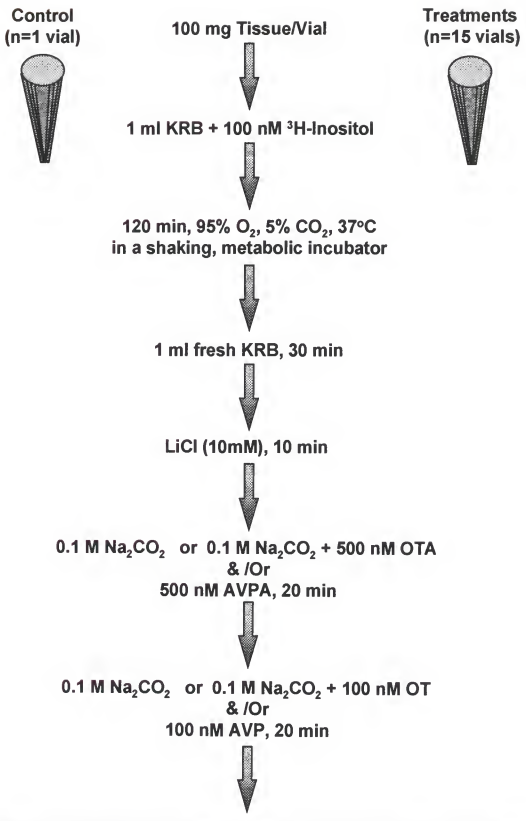
SP 6 mg/ day
1.5 mg/ uterine horn 2X daily

Total protein / injection = 1.5 mg (balanced with SP)

Total volume / injection including saline flush = 3.1 ml

Tissues collected for OTr (Peg method), Er mRNA, Er and Pr protein

Figure 3.6: Schematic of the AVP/OT-stimulated IP assay. Individual treatments listed by vial number are shown in Table 1.


Control
(n=1 vial)
100 mg Tissue/Vial
Treatments
(n=15 vials)
1 ml KRB + 100 nM ^{3}H-Inositol
120 min, 95% O_2, 5% CO_2, 37°C
in a shaking, metabolic incubator
1 ml fresh KRB, 30 min
LiCl (10mM), 10 min
0.1 M Na_2CO_2 or 0.1 M Na_2CO_2 + 500 nM OTA
& /Or
500 nM AVPA, 20 min
0.1 M Na_2CO_2 or 0.1 M Na_2CO_2 + 100 nM OT
& /Or
100 nM AVP, 20 min
Cells were lysed and IP's separated on anion exchange columns

incubated for an additional 30 min. Buffer was replaced again and 20 μl LiCl (0.51 M) was added (to inhibit inositol 1-monophosphatase activity) and tissues incubated 10 min. Following this 10 min incubation, appropriate vials were incubated 15 min with 20 μl KRB + 20 μl OTr antagonist (26 μM in $NaCO_3$; L-365,209; generously supplied by Dr. D.J. Pettibone; Merck, Sharp and Dohme Research Laboratories, West Point, PA), 20 μl KRB + 20 μl AVPr antagonist (26 μM in $NaCO_3$; Peninsula Laboratories, Inc.), or 20 μl OTr antagonist + 20 μl AVPr antagonist . All other vials were balanced with KRB buffer. Following the 15 min incubation, appropriate vials were incubated 20 min with 20 μl oxytocin (5.2 μM in $NaCO_3$; Sigma Chemical Co., St. Louis, MO), or 20 μl AVP (5.2 μM in $NaCO_3$; Sigma Chemical Co., St. Louis, MO). All other vials were balanced with KRB buffer. Following the 20 min incubation, buffer was replaced with ice-cold 15% TCA (w/v) and vials placed on ice for 20-30 min to stop the reaction. The TCA was transferred to boracilicate glass tubes and extracted five times with 5 ml water saturated with diethyl ether. Extracts were discarded each time and the residual diethyl ether was removed from the aqueous phase under a stream of N_2. The samples were neutralized with 25 μl Tris(hydroxymethyl)aminomethane (0.5 M; pH 7.5) and stored at -20°C.

Inositol phosphates (IP_1, IP_2, IP_3) were separated on Dowex columns. Dowex anion-exchange resin (1 x 8 -200; Sigma

Table 1. Arginine Vasopressin/ Oxytocin-induced Inositol Phosphate Metabolism

Vial #	Main Treatment		Antagonist Treatment	
1	Control	- 40μl KRB	None	- 40μl KRB
2	Control	- 40μl KRB	OTrA	- 20μl OTrA + 20μl KRB
3	Control	- 40μl KRB	AVPrA	- 20μl AVPrA + 20μl KRB
4	Control	- 40μl KRB	OTrA + AVPrA	- 20μl OTrA + 20μl AVPrA
5	OT	- 20μl OT + 20μl KRB	None	- 40μl KRB
6	OT	- 20μl OT + 20μl KRB	OTrA	- 20μl OTrA + 20μl KRB
7	OT	- 20μl OT + 20μl KRB	AVPrA	- 20μl AVPrA + 20μl KRB
8	OT	- 20μl OT + 20μl KRB	OTrA + AVPrA	- 20μl OTrA + 20μl AVPrA
9	AVP	- 20μl AVP + 20μl KRB	None	- 40μl KRB
10	AVP	- 20μl AVP + 20μl KRB	OTrA	- 20μl OTrA + 20μl KRB
11	AVP	- 20μl AVP + 20μl KRB	AVPrA	- 20μl AVPrA + 20μl KRB
12	AVP	- 20μl AVP + 20μl KRB	OTrA + AVPrA	- 20μl OTrA + 20μl AVPrA
13	OT + AVP	- 20μl OT + 20μl AVP	None	- 40μl KRB
14	OT + AVP	- 20μl OT + 20μl AVP	OTrA	- 20μl OTrA + 20μl KRB
15	OT + AVP	- 20μl OT + 20μl AVP	AVPrA	- 20μl AVPrA + 20μl KRB
16	OT + AVP	- 20μl OT + 20μl AVP	OTrA + AVPrA	- 20μl OTrA + 20μl AVPrA

Chemical Co.; St. Louis, MO.) was swelled in distilled water and poured into 6 x 0.6 cm columns (n=16). Resin was converted to the formate form by sequential washing with five volumes of 1 M HCl, 1 M NH_4OH, 1 M formic acid, and 0.1 M formic acid in 2 M NH_4 formate. Columns were washed with 15 volumes distilled water and samples were applied to the columns. Sample tubes were rinsed with 0.2 ml distilled water and rinse added to the column. Columns were eluted with 5 x 5 ml distilled water, 3 x 5 ml 25 mM $Na_2B_4O_7$ in 60 mM Na formate, 3 x 5 ml 0.1 M formic acid in 0.2 M NH_4 formate, 3 x 5 ml of 0.1 M formic acid in 0.4 M NH_4 formate and 3 x 5 ml 0.1 M formic acid in 1.0 M NH_4 formate. Each of the elution series were pooled following final 5 ml elution. Inositol, glycerophosphoinositol, IP_1, IP_2, and IP_3 were collect in the five eluents, respectively. Incorporation of [^{3}H]inositol into IPs was determined by liquid scintillation spectrometry.

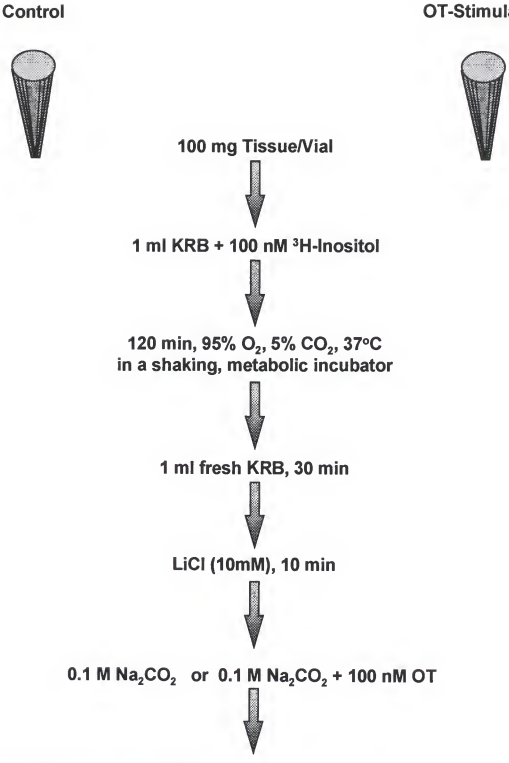
Oxytocin-induced IP metabolism

Oxytocin-induced endometrial IP metabolism was determined as described by Flint *et al.* (1986) and as modified in our laboratory by Vallet and Bazer (1989).

Caruncular tissue from the ipsilateral uterine horn was collected (Experiment 2 and 3) into ice-cold KRB containing 10 mM glucose and 10 μM myo-inositol, minced into approximately 5 mg pieces (Fig. 3.7). Tissue pieces were weighed and 0.1 gm transferred into duplicate glass

scintillation vials and washed with 1 ml ice-cold KRB. One vial served as a control and did not receive oxytocin in the assay and the other had oxytocin added to determine oxytocin-induced IP metabolism. Tissues were incubated under an atmosphere of 95% O_2; and 5% CO_2; at 37°C in a shaking water bath. Vials were re-gassed and returned to the water bath after each change of buffer until the assay was terminated and vials placed on ice. The tissues were incubated for 2 h in 1 ml KRB plus 10 μCi myo-[2-^{3}H]inositol (specific activity 18.9 Ci/mmol.; Amersham, Arlington Heights, IL.). After incubation KRB was removed and fresh KRB without labeled inositol was added and incubated 30 min. Buffer was replaced again with 20 μl LiCl (0.51 M; final concentration = 10 mM) added to inhibit inositol 1-monophosphatase activity and tissues were incubated 10 min. After 10 min, vials were incubated for 20 min with no oxytocin (control vial) or with 5.2 μM oxytocin (oxytocin-induced vial) in 20 μl $NaCO_3$ (0.1 M; final oxytocin concentration = 0 or 100 nM, respectively). After 20 min, buffer was replaced with ice-cold 15% TCA (w/v) and vials placed on ice for 20-30 min to stop the reaction. The TCA was transferred to boracilicate glass tubes and extracted 5 times with 5 ml water saturated diethyl ether. As much diethyl ether as possible was removed with a pipet and discarded each time. Residual diethyl ether remaining after the final wash was removed from the aqueous phase

Figure 3.7: Schematic of the OT-stimulated IP metabolism assay.


Control
OT-Stimulated
100 mg Tissue/Vial
1 ml KRB + 100 nM ^{3}H-Inositol
120 min, 95% O_2, 5% CO_2, 37°C
in a shaking, metabolic incubator
1 ml fresh KRB, 30 min
LiCl (10mM), 10 min
0.1 M Na_2CO_2 or 0.1 M Na_2CO_2 + 100 nM OT
Cells were lysed and IP's separated on anion exchange columns

under a stream of N_2. The samples were neutralized with 25 μl Tris(hydroxymethyl)aminomethane (0.5 M; pH 7.5) and stored at -20°C.

Inositol phosphates were separated on Dowex columns as described previously in the AVP/OT-induced IP metabolism section.

Endometrial Oxytocin Receptor Assay

Filter procedure

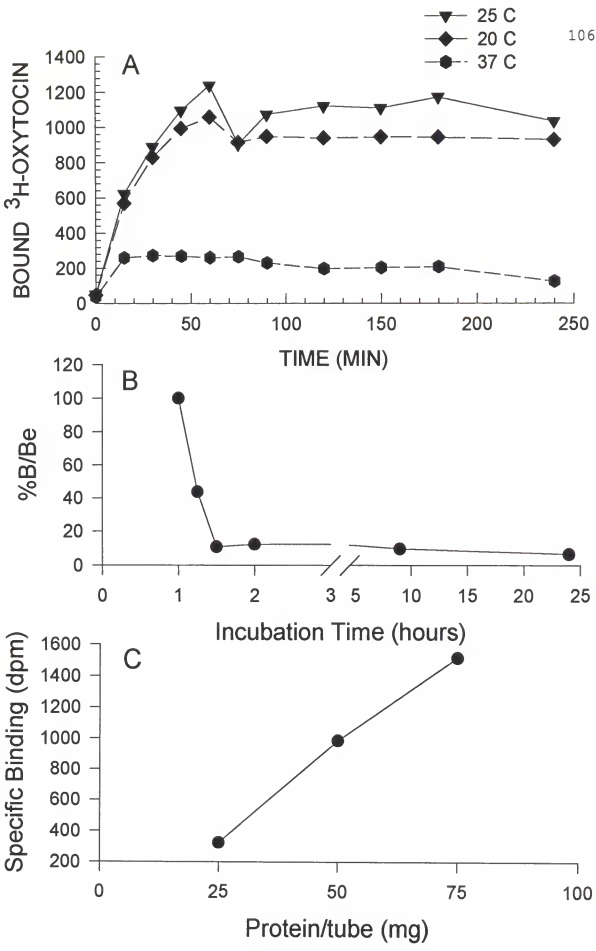
Concentrations of endometrial OTr were determined in Experiment 2 and 3 by the filter method (Sheldrick and Flint, 1985). Tissues previously frozen at -80°C were removed from the freezer and maintained on ice while approximately 1.5 gm of tissue was removed. The remainder of the tissue was returned to the freezer. While frozen, approximately 1 gm of tissue was weighed and minced with a razor blade into fine pieces. The minced tissue was transferred to a 50 ml conical tube maintained on ice and rinsed with 5 ml homogenization buffer (25 mM Tris-HCl, 250 mM Sucrose, 1 mM EDTA; pH 7.4 at 4°C). Buffer was replaced with 10 ml homogenization buffer and endometrium homogenized for 5 sec with a polytron homogenizer (Brinkman Instruments, Westbury, NY) at a speed setting of 8. The homogenate was filtered through two layers of gauze into a chilled ground glass homogenizer, and further homogenized by 10 strokes with a ground glass homogenizer. The homogenate was

decanted into a clean 50 ml conical tube, and the homogenizer rinsed with 2 ml of homogenization buffer which was added to the conical tube. Homogenates were centrifuged at 3,000 x g for 10 min at 4°C. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 196,000 x g for 90 min. After centrifugation the supernatant was discarded, the pellet rinsed twice with 25 mM Tris-HCl (pH 7.4 at 4°C) and 1 ml of this buffer then used to resuspend the membrane preparation. Membrane preparations were transferred to polypropylene tubes, a 200 µl aliquot was removed for protein determination by bicinchoninic acid assay (Smith *et al.*, 1985), and the remainder was divided equally into two separate tubes for storage (-80°C) until assayed.

The assay was validated by measuring the number of receptors in a pool of endometrium collected from six ewes in estrus. Receptor binding was maximal at 60 min at 25°C. At membrane protein concentrations of 25, 50 and 75 mg, there was a linear increase in receptor binding (Fig. 3.8). There was no detectable binding of FSH, LH, or roIFNτ by the membrane preparations when added to the assay (data not shown).

The final assay was with membrane preparations (50 µg/100 µl) incubated for 60 min at 25°C with 5 fmol to 16 pmol [tyrosyl-2,6-3H]-OT (New England Nuclear, Boston, MA; specific activity 37.1 Ci/mmol) in 100 µl 25mM Tris- HCl, 20

Figure 3.8. Validation of the OT binding assay (filter method). Percent specific binding of OT was measured in endometrial pools obtained from estrous ewes. A) temperature validation. Binding was determined at various times (0 to 240 min) following incubation at 20°C (diamonds), 25°C (triangles) and 39°C (heptagon). Binding was maximal at 60 min and 25°C. B) dissociation kinetics. Membrane protein (50μg) was incubated with 3H-OT for 60 min at 25°C. At equilibrium, 1 μg of radioinert OT was added and the samples assayed for radioactivity bound at the indicated times. C) protein validation. Specific binding was determined using increasing concentrations of membrane protein. There was a linear increase in OTr binding when membrane concentrations of 25, 50, and 75 mg/ml were examined.


25 C
20 C
37 C
A
BOUND ^{3}H-OXYTOCIN
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
250
TIME (MIN)
B
%B/Be
120
100
80
60
40
20
0
0
1
2
3
5
10
15
20
25
Incubation Time (hours)
C
Specific Binding (dpm)
1600
1400
1200
1000
800
600
400
200
0
25
50
75
100
Protein/tube (mg)

mM $MnCl_2$, 0.2% BSA, and 0.02% NaN_3. Non-specific binding was estimated in the presence of 800 pM radioinert oxytocin. Following incubation, tubes were placed on ice and 2 ml 25 mM Tris-HCl, 10 mM $MnCl_2$, 0.1% BSA, and 0.01% NaN_3 was added. Receptor fractions were collected onto 0.2 μm GVWP filters (Millipore Corp, Bedford, MA.). Filters were rinsed twice, placed into 5 ml plastic scintillation vials with 4.5 ml Scintiverse II (Fisher), allowed to equilibrate 4 h and receptor-bound [^{3}H]-oxytocin quantified by liquid scintillation spectrometry. Number and K_d of receptors were determined by Scatchard analysis using LIGAND (Munson and Rodbard, 1980).

PEG assay

Concentrations of endometrial OTr were determined in Experiment 4 and 5 by a PEG assay method modified from procedures reported by Sernia *et al.* (1991) and Lau *et al.* (1992).

Tissues frozen at -80°C were removed from the freezer and maintained on ice while approximately 1.5 gm tissue was removed. The remainder of the tissue was returned to the freezer. While frozen, 1 gm of tissue was weighed and minced with a razor blade into very fine pieces. The minced tissue was transferred to a 50 ml conical tube maintained on ice and rinsed with 5 ml homogenization buffer (50 mM Tris-HCl, 250 mM sucrose, 4 mM EDTA; pH 7.4 at 4°C). Buffer was

replaced with 10 ml homogenization buffer and endometrium was homogenized for 5 sec with a polytron homogenizer (Brinkman Instruments, Westbury, NY) at a speed setting of 8. The homogenate was filter through two layers of gauze into a chilled ground glass homogenizer, and further homogenized 10 strokes with the ground glass homogenizer and poured into a clean 50 ml conical tube. The ground glass homogenizer was rinsed with 2 ml homogenization buffer and the rinse added to the conical tube. Homogenates were centrifuged at 3,000 x g for 10 min and 4°C. Supernates were transferred to ultracentrifuge tubes and centrifuged at 196,000 x g for 90 min. After centrifugation the supernate was discarded, the pellet rinsed twice with 50 mM Tris-HCl (pH 7.4 at 25°C) and 1 ml of this buffer was replaced to resuspend the membrane preparation. Membrane preparations were transferred to polypropylene tubes, a 200 μl aliquot was removed for protein determination by bicinchoninic acid assay, and the remainder was divided equally into two tubes for storage (-80°C) until assayed.

The assay was validated by measuring the number of receptors in a pool of endometrium collected from four ewes at estrus. Receptor binding was maximal at 2 h and 25°C in 20% PEG (Fig. 3.9). There was a linear increase in receptor binding when membrane protein concentrations of 25, 50, 100, and 200 μg/ml were examined (Fig. 3.10). There was no

detectable binding of FSH, LH, or roIFNτ to membrane preparations (data not shown).

The endometrial OTr within the membrane preparations were measured by radioreceptor assay. This assay consisted of 100 μl (200 μg) membrane preparation from a frozen aliquot, 100 μl (0.321 pM) [tyrosyl-2,6-3H]-oxytocin(New England Nuclear, Boston, MA; specific activity 39.0 Ci/mmol) and 100 μl assay buffer (50 mM Tris-HCl, 20 mM $MnCl_2$, 0.3% BSA (w/v) and 0.2% NaN_3(w/v); pH 7.6 at 25°C) containing 0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, and 80 pM radioinert oxytocin. The membrane preparations were incubated at 25°C for 2 h. Bound oxytocin was precipitated by the addition of 100 μl γ-globulin (8 mg/ml; pig derived; Sigma Chemical Co., St Louis, MO) and 1 ml 20% (w/v) PEG followed by centrifugation at 3,000 x g for 10 min. The precipitate was dissolved in 400 μl 50 mM Tris-HCl and precipitated a second time with 1 ml 20% PEG followed by centrifugation at 3,000 x g for 10 min. The precipitate was dissolved in 400 μl 50 mM Tris-HCl, to which 4 ml scintillation fluid (Scintiverse II; Fisher Scientific, Inc.) was added. After equilibrating for 1 h, samples were quantified by liquid scintillation spectrometry.

Statistical Analysis

Data were subjected to least squares ANOVA using the GLM procedure of SAS (SAS Institute, 1985). Data for IP

Figure 3.9. Temperature validation of the polyethylene glycol (PEG) binding assay for the measurement of endometrial OTr. Percent specific binding of OT was measured in endometrial pools obtained from estrous ewes. Binding was determined every 30 min though 4.5 h and again at 24 h following incubation at 25°C (squares) and at 39°C (triangles). Binding was maximal at 2 h and 25°C.

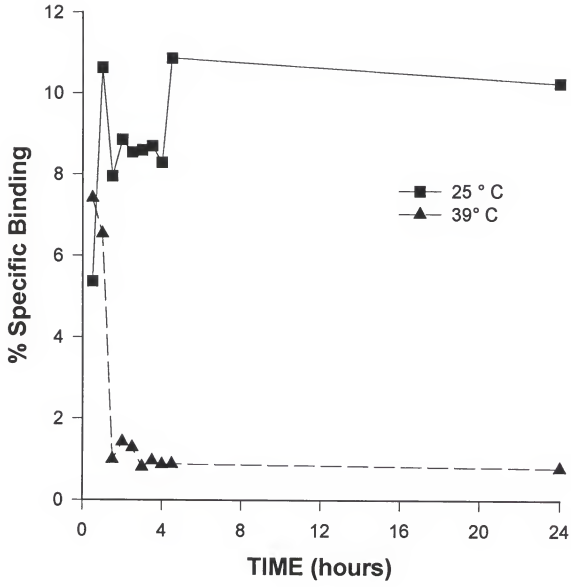
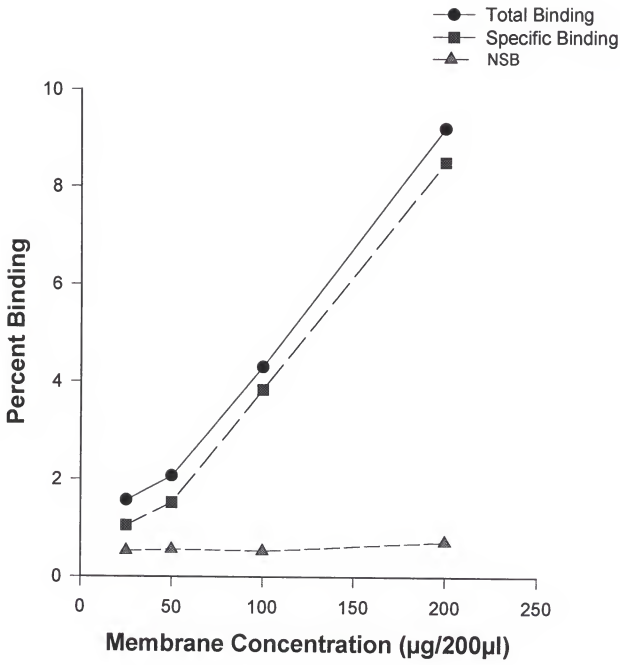

12
10
8
6
4
2
0
% Specific Binding
25 ° C
39° C
0
4
8
12
16
20
24
TIME (hours)

Figure 3.10. Protein validation of the polyethylene glycol (PEG) binding assay for the measurement of endometrial OTr. Percent specific binding (squares), total binding (circles) and nonspecific binding (NSB; triangles) of OT was measured in endometrial pools obtained from estrous ewes. Binding was determined using increasing concentrations of membrane protein. There was a linear increase in OTr binding at membrane concentrations of 50, 100, and 200 μg/ml.


Total Binding
Specific Binding
NSB
Percent Binding
10
8
6
4
2
0
0
50
100
150
200
250
Membrane Concentration (µg/200µl)

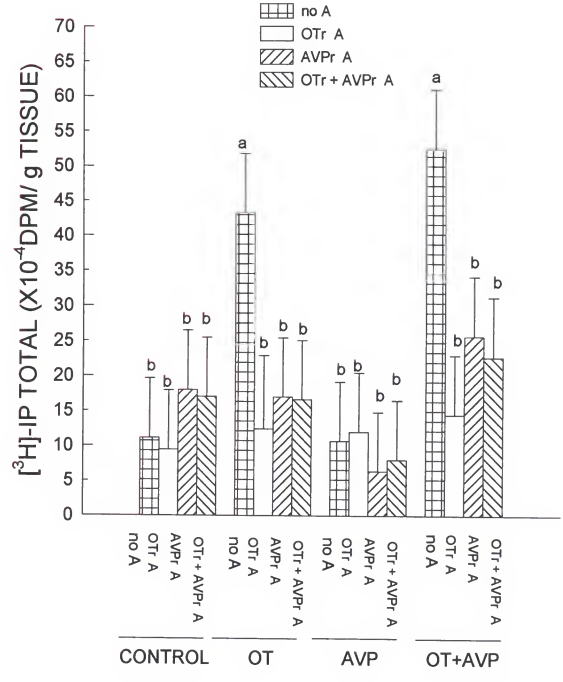
metabolism analysis were analyzed untransformed and log-transformed to alleviate statistical problems associated with heterogeneity of variance. Least squares means and standard errors were obtained using the least squares means statement of the GLM procedure. Ewes were nested within treatment for Experiments 2 through 5. All tests of hypothesis were performed using the appropriate error terms according to the expectation of the mean squares (Snedecor and Cochran, 1980). Data presented are least squares means ± SEM.

Results

Inositol Phosphate Metabolism

In Experiment 1, there was no detectable IP metabolism induced by AVP stimulation of Day 16 ovine endometrium. As indicated in Fig. 3.11, tissue exposed to AVP (vials 9-12; see Table 1) did not respond with an elevation in IP metabolism over basal levels (vial 1). Both the OTr antagonist and the AVP receptor antagonist cross-reacted with the OTr to prevent an increase in IP metabolism induced by oxytocin. Also, after treatment with either OTr antagonist or AVP receptor antagonist alone there was no detectable stimulation of IP metabolism over that of controls. There was no synergistic effect between oxytocin and AVP (vial 13) on IP metabolism when compared to the

Figure 3.11. IP metabolism by endometrium from estrous ewes in response to OT, AVP, and OTrA and AVPrA (Experiment 1). Tissues from Day 0 ewes were pretreated with no antagonist (control; no A), OTrA, AVPrA or both the OTrA and the AVPrA. Tissues were subsequently exposed to control medium alone, OT, AVP, or OT+AVP. Bars with the same letters are not different (P>0.05).


no A
OTr A
AVPr A
OTr + AVPr A
[3H]-IP TOTAL (X10-4DPM/ g TISSUE)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
a
b
b
b
b
b
b
b
b
b
b
a
b
b
b
no A
OTr A
AVPr A
OTr + AVPr A
CONTROL
OT
AVP
OT+AVP

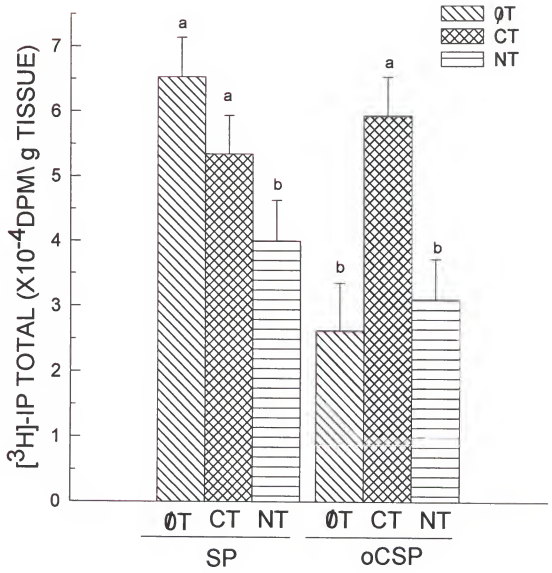
level of IP metabolism induced by oxytocin alone (vial 5). These results indicate that Class I AVP receptors are either not present in Day 16 ovine endometrium or that they are present in inadequate numbers to permit AVP stimulation of IP metabolism. Results also indicate that there is no cross-reactivity between AVP and oxytocin to stimulate OTr to increase IP metabolism. Therefore, it appears that the increase in IP metabolism in these experiments was due to oxytocin stimulation of OTr.

Oxytocin induced IP metabolism

Oxytocin-induced IP metabolism in endometrial tissues of ewes treated with SP, the synthetic NT peptide of oIFNτ, or oCSP is shown in Fig. 3.12. Intrauterine injection of NT alone blocked oxytocin-induced IP metabolism after oxytocin stimulation of endometrial tissues. Treatment of ewes with NT + oCSP also attenuated oxytocin-induced metabolism of IPwithin endometrial tissues, but the effect was not additive. Interestingly, the CT had no effect on oxytocin-induced IP metabolism when given alone, but when given in conjunction with oCSP it negated the inhibitory effect of oCSP on oxytocin-induced IP metabolism.

The results of oxytocin-induced IP metabolism for Experiment 3 are shown in Fig. 3.13. Inositol phosphate metabolism in endometrial tissues from ewes treated with SP was higher ($P<0.05$) than in tissues from ewes treated with

Figure 3.12. *In vitro* oxytocin induced endometrial IP metabolism in ewes receiving intrauterine injections of SP, oCSP, synthetically produced peptides of oIFN$_t$, NT and CT, or combinations of the proteins and peptides (Experiment 2). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT + 1.0 mg SP, 0.5 mg CT + 1.0 mg SP, 0.75 mg oCSP + 0.75 mg SP, 0.75 mg oCSP + 0.5 mg NT or 0.75 mg oCSP + 0.5 mg CT. Treatments began on Day 12 and continued through the morning of Day 16. Zero (0) Pep indicates ewes were treated with SP or oCSP only. Bars that share letters are not different (P>0.05).


[3H]-IP TOTAL (X10-4DPM\ g TISSUE)
0
1
2
3
4
5
6
7
a
a
b
b
a
b
ØT
CT
NT
ØT CT NT
ØT CT NT
SP
oCSP

NT (aa, 1-37), Pep 2 (aa, 34-64), Pep 3 (aa, 62-92), Pep 4 (aa 90-122) or roIFNτ. Inositol phosphate metabolism within endometrial tissues collected from ewes treated with Pep 5 (aa, 119-150) was not different from that for SP-treated ewes. Furthermore, IP metabolism within endometrium was not different for treatment with Pep 5 *vs.* NT, Pep 2-4 or roIFNτ.

These results indicate that peptides, corresponding to amino acids 1-122, blocked oxytocin-induced IP metabolism in Day 16 ovine endometrium. There was, however, no effect of Pep 5, corresponding to amino acids 119-150, on IP metabolism.

Endometrial Oxytocin Receptor Assay

The results from Experiment 2 showing endometrial OTr concentrations expressed in fmol/mg protein are presented in Fig. 3.14. The main treatment effects of oCSP and SP are shown in Fig. 3.14A, while the peptide main effects and the treatment by peptide interactions are shown in Fig. 3.14B, and Fig. 3.14C, respectively. Regardless of peptide treatment, intrauterine injection of oCSP blocked the formation of endometrial OTr (Fig. 3.14A). Intrauterine injection of NT alone or with oCSP almost completely attenuated development of endometrial OTr (Fig. 3.14C). Intrauterine injection of CT also attenuated the formation of endometrial OTr, but was not as effective as NT (Fig.

3.14C). The CT treatment, when given with SP, was only half as effective in blocking OTr formation in endometrial tissues as NT.

Results from Experiment 3 are presented in Fig. 3.15. Intrauterine injections of NT, Pep 2, Pep 3, or Pep 4 almost completely attenuated the formation of endometrial OTr, as did treatment with roIFNτ. In several instances receptor numbers were undetectable in the assay. Treatment of ewes with Pep 5 blocked OTr formation compared to controls with about a 2-fold difference in endometrial receptor number between that for SP-treated ewes and ewes treated with Pep 5. However, Pep 5 was not as effective in blocking receptor formation as were the other peptides or roIFNτ.

The endometrial OTr concentration results expressed as percent specific binding for Experiment 4 are shown in Fig. 3.16. Results are expressed as specific binding because receptor numbers were so low in the NT and roIFNτ treated groups that receptor number determination by Scatchard analysis was not possible. However, specific binding was determined to be an acceptable response in the determination of differences between treatments. The OTr concentrations within endometrial tissues of ewes treated with NT and roIFNτ were not different from each other, but they were almost 10-times less than for ewes treated with SP.

Figure 3.13. Endometrial IP metabolism in ewes receiving intrauterine injections of SP, synthetically produced peptides of oIFN$_t$, or roIFN$_t$ (Experiment 3). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.25 μg roIFN$_t$, or 0.5 mg each of synthetic peptide NT, 2, 3, 4, or 5. The total protein concentrations per treatment were balanced to 1.5 mg with SP. Treatments began on Day 12 and continued through the morning of Day 16. Bars with the same letters are not different ($P>0.05$).

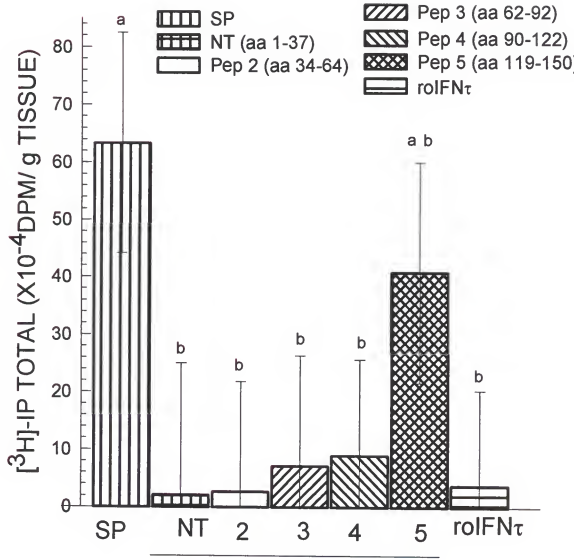
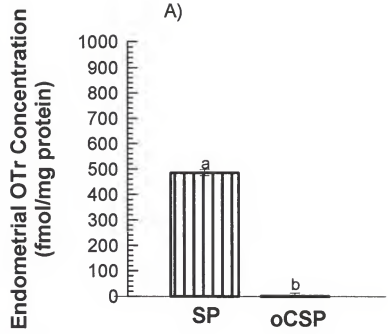
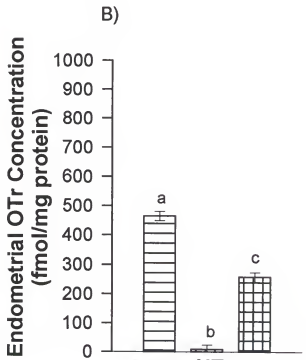

SP
NT (aa 1-37)
Pep 2 (aa 34-64)
Pep 3 (aa 62-92)
Pep 4 (aa 90-122)
Pep 5 (aa 119-150)
roIFNτ
[3H]-IP TOTAL (X10-4DPM/ g TISSUE)
0
10
20
30
40
50
60
70
80
a
b
b
b
b
a b
b
SP
NT
2
3
4
5
roIFNτ

Figure 3.14. Endometrial OTr concentrations in ewes receiving intrauterine injections of SP, oCSP, synthetically produced peptides to domains of oIFN$_\tau$, or combinations of the proteins and peptides (Experiment 2). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT + 1.0 mg SP, 0.5 mg CT + 1.0 mg SP, 0.75 mg oCSP + 0.75 mg SP, 0.75 mg oCSP + 0.5 mg NT or 0.75 mg oCSP + 0.5 mg CT per uterine horn. Treatments began on Day 11 and continued through the morning of Day 16. A, main effects of treatment. B, main effects of peptide treatment. C, effects of treatment by peptide interaction. Bars with the same letters are not different ($P>0.05$).


A)
Endometrial OTr Concentration (fmol/mg protein)
1000
900
800
700
600
500
400
300
200
100
0
a
b
SP
oCSP


B)
Endometrial OTr Concentration (fmol/mg protein)
1000
900
800
700
600
500
400
300
200
100
0
a
b
c
ØPep NT CT

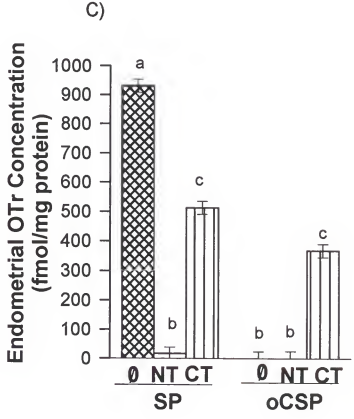

C)
Endometrial OTr Concentration (fmol/mg protein)
1000
900
800
700
600
500
400
300
200
100
0
a
b
c
b b
c
Ø NT CT
SP
Ø NT CT
oCSP

Figure 3.15. Endometrial OTr concentrations in ewes receiving intrauterine injections of SP, synthetic peptides to specific domains of oIFN$_\tau$, or roIFN$_\tau$ (Experiment 3). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 25 μg roIFN$_\tau$, or 0.5 mg each of synthetic peptide NT, 2, 3, 4, or 5 per uterine horn. The total protein concentrations per treatment were balanced to 1.5 mg with SP. Treatments began on Day 12 and continued through the morning of Day 16. Bars with the same letters are not different ($P>0.05$).

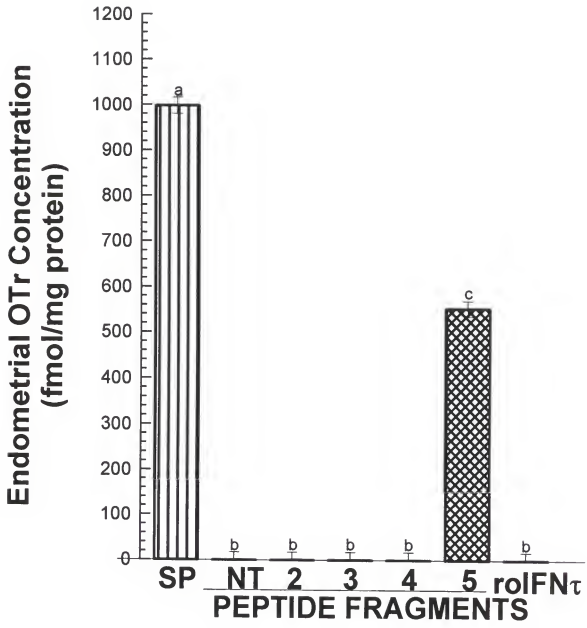
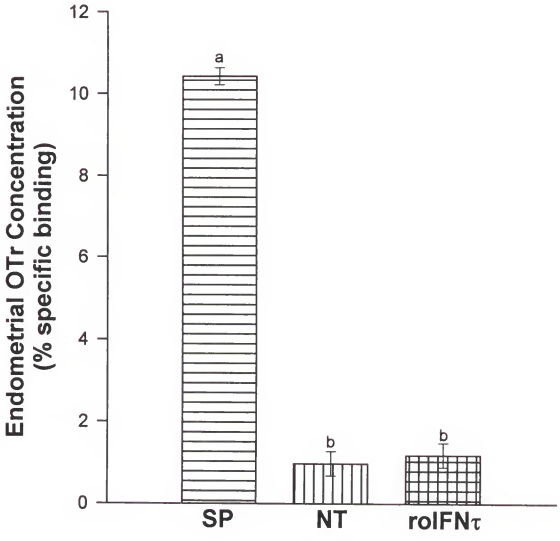

Endometrial OTr Concentration
(fmol/mg protein)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
a
b
b
b
b
c
b
SP
NT
2
3
4
5
roIFNτ
PEPTIDE FRAGMENTS

Figure 3.16. Endometrial OTr concentrations in ewes receiving intrauterine injections of SP, NT, or roIFN$_\tau$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 50 μg roIFN$_\tau$ or 0.5 mg NT per uterine horn. The total protein concentrations per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Days 13 and 15. Bars with the same letters are not different ($P>0.05$).

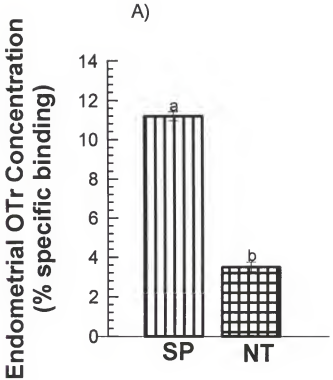

Endometrial OTr Concentration
(% specific binding)
12
10
8
6
4
2
0
a
b
b
SP
NT
roIFNτ

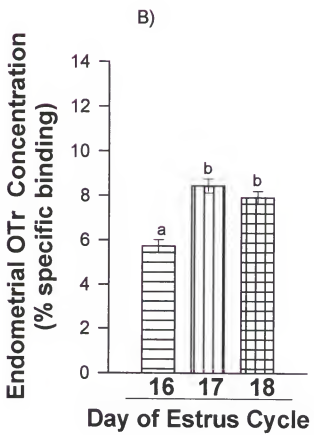
Endometrial OTr concentrations in tissues from ewes collected on Days 16, 17 or 18 after intrauterine injections with either SP or NT (Experiment 5) are shown in Fig. 3.17A, B and C. Treatment with NT attenuated formation of OTr in ewes on all days examined, compared to ewes treated with SP. Receptor concentrations were lowest in ewes treated with NT and collected on Day 16. There was a 4-fold increase in OTr numbers from Day 16 to Day 17 in ewes treated with NT; however, this number went down slightly from Day 17 to Day 18. In ewes treated with SP there was a steady increase in OTr concentrations. While values were not different between Day 16 and Day 17, the increase between Day 16 and Day 18 was different. The OTr concentrations in tissues collected from ewes treated with SP and collected on Day 17 were intermediate to values on the other two days.

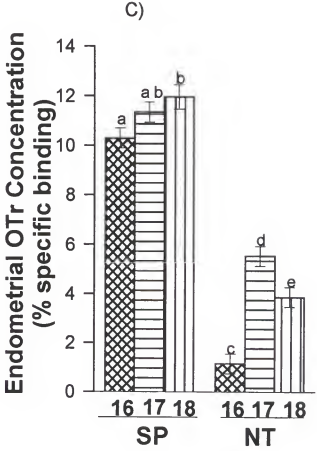
Discussion

Ovine IFNτ, the conceptus secreted maternal pregnancy recognition factor (Godkin *et al.*, 1984b; Valle*et al.*, 1988), is assumed to bind to high affinity (Godkin *et al.*, 1984a) Type I IFN receptors (Stewert *et al.*, 1987) which are distributed throughout endometrial tissues of the ewe (Knickerbocker and Nisewinder, 1989). Trophoblast IFNs are biologically similar to other Type I IFNs and like the other IFNτs, oIFNτ displays both antiviral and antiproliferative properties (Pontzer *et al.*, 1988).

Figure 3.17. Endometrial OTr measurements, expressed as percent specific binding, in ewes receiving intrauterine injections of SP or NT (Experiment 5). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP or 0.5 mg NT + 1.0 mg SP per uterine horn. Treatments began on Day 11 and continued through the morning of Days 16, 17 or 18 when tissues were collected. A, main effects of treatment. B, main effects of day. C, effects of treatment by day. Bars with the same letters are not different ($P>0.05$).


A)
Endometrial OTr Concentration
(% specific binding)
14
12
10
8
6
4
2
0
a
b
SP
NT


B)
Endometrial OTr Concentration
(% specific binding)
14
12
10
8
6
4
2
0
a
b
b
16 17 18
Day of Estrus Cycle


C)
Endometrial OTr Concentration
(% specific binding)
14
12
10
8
6
4
2
0
a
a b
b
c
d
e
16 17 18
16 17 18
SP
NT

Synthetic NT peptides and CT peptides of oIFNτ (Pontzer *et al.*, 1991) as well as two internal peptides (aa 62-92 and aa 119-150) inhibit oIFNτ receptor binding and antiviral activity in a dose dependent manner (Pontzer *et al.*, 1994) indicating specific competition between those specific peptides and oIFNτ. The NT has no effect on antiviral activity of IFNα. Collectively, these findings indicate that the NT acts through a novel domain of the oIFNτ receptor while the other peptides may act through another more common domain of the Type I receptor. This may explain the unique actions of oIFNτ. Ovine IFNτ and other Type I IFNs increase endometrial protein production dramatically (Gross *et al.*, 1988b; Sharif *et al.*, 1989; Ashworth and Bazer, 1989).

Our current working hypothesis for pregnancy recognition, supported by recent work by Spencer *et al.* (1995a, 1995b, 1996), is that in the pregnant ewe, oIFNτ acts to prevent luteolysis, by preventing transcription of the Er gene which in turn prevents the formation of endometrial OTr on the luminal and superficial glandular epithelium. The mechanism by which oIFNτ alters OTr up-regulation and reduces endometrial responsiveness to oxytocin is not known, but is most likely due to negative-acting transcriptional factors such as interferon regulatory factor-2. In the cyclic ewe, long term exposure to progesterone down-regulates Pr allowing expression of Er and

OTr (McCracken *et al.*, 1984;) especially in the luminal endometrium and superficial glandular epithelium (Spencer and Bazer, 1995). In the pregnant ewe, progesterone down-regulates endometrial Pr in luminal endometrial and superficial glandular epithelium, just as it does in the cyclic ewe, but unlike in the cyclic ewe, Er and OTr are undetectable in these tissues (Spencer *et al.*, 1995). Pr and Er are detectable only in low levels in the stroma and deep glandular epithelium in pregnant ewes. Therefore, it is believed that oIFNτ actions are inhibitory to Er gene expression. By this action, OTr formation is attenuated and the positive feedback loop of ovarian oxytocin and pulsatile uterine $PGF_{2\alpha}$ is blocked in the pregnant ewe. Without the effects of pulsatile $PGF_{2\alpha}$ to regress the CL, progesterone production is maintained, and it can act on Pr-positive stroma and deep glandular epithelium to continue the suppression Er and OTr genes.

Oxytocin stimulates endometrial production of $PGF_{2\alpha}$ (Fairclough *et al.*, 1984; Vallet *et al.*, 1988, Chapter 4) and IP metabolism in oxytocin-stimulated endometrium of cyclic and SP-treated ewes (Mirando *et al.*, 1990). These results show that oxytocin stimulates uterine secretion of $PGF_{2\alpha}$ during luteolysis via activation of the IP/diacylglycerol signal transduction system. Oxytocin's specific action through the IP/diacylglycerol signal transduction system is further indicated by results of the

present study with oxytocin, AVP and their antagonists which indicate that IP metabolism is due to effects of oxytocin acting via OTr and not through the AVP receptor in the ewe.

IP metabolism, however, is an indirect measure of OTr in endometrial tissues. While it is not currently known how oIFNτ blocks expression of Er and OTr genes, Er up-regulation is apparently required for OTr gene expression in endometrial luminal epithelium (McCracken *et al.*, 1980; Vallet *et al.*, 1990; Silvia *et al.*, 1991; Beard *et al.*, 1994; Spencer *et al.*, 1995a, 1995b, 1996). Estrogen is necessary for development of the luteolytic mechanism and, in the absence of estrogen, oxytocin is unable to initiate luteolytic PGF2α release (Zhang *et al.*, 1992). Furthermore, treatment of cyclic ewes with estrogen up-regulates OTr expression and initiates premature luteolysis (Hixon *et al.*, 1987). Intrauterine injections of oCSP (Mirando *et al.*, 1993; Chapters 3 and 5) and roIFNτ (Spencer *et al.*, 1995; 1996; Chapters 3 and 5) blocks estradiol-induced Er and OTr expression. Results of the present study indicate that NT and oIFNτ are equally affective in attenuating formation of endometrial OTr. The ability of NT to effectively block up-regulation of endometrial OTr continued for up to 48 h after the last intrauterine injection was administered. However, treatment with CT of oIFNτ did not attenuate the increase in endometrial OTr. These findings support those of Pontzer

et al. (1994) which indicated that the NT of oIFNτ has a unique interaction with the Type I oIFNτ IFN receptor in endometrium.

CHAPTER 4
THE EFFECTS OF RECOMBINANT OVINE INTERFERON *TAU* AND SYNTHETIC PEPTIDE DOMAINS OF INTERFERON *TAU* ON OXYTOCIN-STIMULATED PROSTAGLANDIN-F METABOLITE CONCENTRATIONS IN PLASMA OF EWES.

Introduction

Prostaglandin $F_{2\alpha}$ produced by endometrial luminal epithelium causes luteal regression in ewes (McCracken *et al.*, 1972). Luteal regression is achieved by the high amplitude, short duration pulsatile secretion of $PGF_{2\alpha}$ (Thornburn *et al.*, 1983; Barcikowski *et al.*, 1974; Flint and Sheldrick, 1983; Zarco *et al.*, 1984, 1988) initiated by the pulsatile release of oxytocin (Flint *et al.*, 1986). Together uterine $PGF_{2\alpha}$ and ovarian oxytocin form a positive loop, with 50% (Walker *et al.*, 1997) to 97% of all episodes of uterine $PGF_{2\alpha}$ secretion being coincidental with pulses of oxytocin in the blood (Hooper *et al.*, 1987). Estrogen enhances endometrial secretion of $PGF_{2\alpha}$ in response to oxytocin (McCracken *et al.*, 1984). Endometrial OTr are increased by estrogen (Hixon and Flint, 1987) and although, chronic progesterone treatment will allow OTr receptor formation after Pr are down-regulated, endometrial OTr formation is stimulated

optimally by a regime of progesterone followed by estrogen (Vallet *et al.*, 1990, see Spencer *et al.*, 1996). During early pregnancy the pulsatile pattern of $PGF_{2\alpha}$ secretion is attenuated (Thornburn *et al.*, 1973; Barcikowski *et al.*, 1974; Moore *et al.*, 1982; Hooper *et al.*, Zarco *et al.*, 1988a) by disruption of the oxytocin/$PGF_{2\alpha}$ positive feedback loop. The normal response of increased $PGF_{2\alpha}$ secretion in response to exogenous estrogen (Fincher *et al.*, 1986) and oxytocin (Fairclough *et al.*, 1984) also is blocked during early pregnancy. The attenuation of the loop in the pregnant ewe is due primarily to a blockage of endometrial OTr formation seen in the cyclic ewe just prior to luteolysis (McCracken *et al.*, 1984; Sheldrick and Flint, 1985). It is this reduction in endometrial Er and OTr which is responsible for the decreased endometrial responsiveness to oxytocin during early pregnancy in ewes (McCracken *et al.*, 1984; Fairclough *et al.*, 1984; Mirando *et al.*, 1990a, 1990b)

Ovine IFNτ is the primary protein component of oCSP and the antiluteolytic protein secreted by the conceptus (Godkin *et al.*, 1992; Vallet *et al.*, 1988; see Bazer *et al.*, 1991; Ott *et al.*, 1993a). Intrauterine injection of oCSP, highly purified oIFNτ or roIFNτ are effective in blocking the luteolytic mechanism (Vallet *et al.*, 1989b). Therefore, these studies were undertaken to determine; (1) if roIFNτ is as effective as native oIFNτ in blocking oxytocin-stimulated

$PGF_{2\alpha}$ secretion (as determined by measuring the metabolite of $PGF_{2\alpha}$, PGFM) and (2) if peptides representing major domains of oIFNτ elicited the same response as roIFNτ.

Materials and Methods

Animals

Ewes of primarily Rambouillet breeding were checked for estrous behavior daily at 07:30 AM for 20 min with vasectomized males. Ewes which had exhibited at least two normal estrous cycles (16 to 17 days in length) were assigned to experimental groups. Ewes in this study were housed at the Sheep Research Center, Texas A&M University.

Protein And Peptide Preparation

Recombinant ovine interferon *tau* preparation

Recombinant ovine interferon *tau* was produced and prepared as described in Chapter 3.

Serum protein preparation

Serum proteins were prepared as described in Chapter 3.

Synthetic peptide production

A synthetic peptide corresponding to the NT portion of roINFτ was produced as described in Chapter 3.

Experimental Design

Experimental design for Experiment 4 is discussed in Chapter 3.

Prostaglandin-F metabolite assay

Plasma, processed from jugular vein blood samples obtained from ewes after they had been challenged with oxytocin in Experiment 4, was assayed for PGFM. The PGFM assay had been validated previously for sheep by Fincher *et al.* (1986) and cross-reactivities of the antibody with other prostaglandins had been determined (Guilbault *et al.*, 1984; Knickerbocker *et al.*, 1986). Plasma used for preparation of all standards was obtained from banamine-treated sheep (100 mg/injection, two injections 12 h apart) to inhibit prostaglandin synthesis. Each standard was measured in triplicate and each unknown in duplicate. The standard curve measured PGFM at 50, 25, 10, 0.5, 0.25, 0.10, 0.05, 0.025, 0.01, and 0.005 ng/ml. The PGFM was measured by incubating (15 min at 25°C) 200 μl plasma in 100 μl Tris-HCl (0.05 M; pH 7.5 at 4°C) with 100 μl 0.5% human γ-globulin. After incubation, 100 μl PGFM anti-serum (goat anti-PGFM 23; 1/6,000; Supplied by Dr. Ken Kirton, Upjohn Co., Kalamazoo MI) was added and allowed to incubate 30 min at 25°C. Radiolabled PGFM (100 μl ^{3}H-PGFM; ~18,000 dpm) was added, incubated 1 h at 25°C, and then overnight at 4°C. Bound

radiolabled PGFM was precipitated with 750 µl PEG 8000 (40 % w/v; 4°C) and centrifuged 30 min at 3,000 x g at 4°C. The supernate was discarded, the pellet redissolved in 750 µl Tris-HCl (0.05 M pH 7.5 at 4°C), bound PGFM reprecipitated with 750 µl PEG, and the sample centrifuged for 30 min at 3,000 x g at 4°C. The supernate was again discarded and the pellet resuspended in 1 ml Tris-HCl. The solution was transferred to plastic scintillation vials and 4 ml scintillation cocktail (Bio-HP) added. Scintillation vials were allowed to equilibrate 1 h and counted. The results were expressed in pg/ml PGFM in plasma.

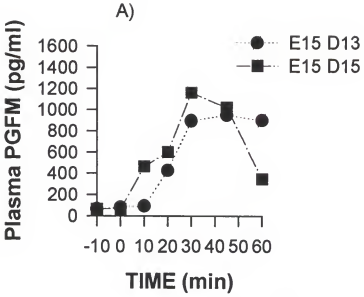
Statistical Analysis

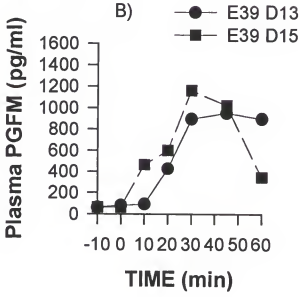
Data were subjected to least squares ANOVA using the GLM procedure of SAS (SAS Institute, 1985). Tests of homogeneity of regression were performed to detect differences in patterns in PGFM secretion over the days examined for the treatment groups. Least squares means and standard errors were obtained using the least squares means statement of the GLM procedure. Ewes were nested within treatment and all tests of hypothesis were performed using the appropriate error terms according to the expectation of the mean squares (Snedecor and Cochran, 1980).

Results

Results of individual ewe PGFM responses are shown in Fig. 4.1 through 4.3. Fig. 4.1 shows the PGFM response after oxytocin stimulation in individual ewes treated with OT on Days 13 and 15. Two SP-treated ewes responded to oxytocin challenge on both Day 13 and Day 15. Figures 4.2 and 4.3 indicate responses of individual ewes treated with NT and roIFNτ, respectively. Ewes that received intrauterine injections of NT or roIFNτ had small or undetectable increases in PGFM on Day 13 in response to the oxytocin challenge. On Day 15 two of the roIFNτ-treated ewes also were unresponsive to oxytocin challenge. Mean plasma PGFM concentrations after oxytocin challenge for all treatments are shown in Fig. 4.4. Plasma PGFM concentrations were lower ($P<0.02$) in ewes treated with roIFNτ and NT compared to SP on both Day 13 and Day 15 (Fig. 4.4). The mean PGFM response to oxytocin was lower on Day 13 than Day 15 for all treatments (Fig. 4.5). The mean PGFM responses (treatment*day) for all treatments are shown in Fig. 4.6. Analysis of regression curves (treatment*day*time; Fig. 4.7) indicated that attenuation of PGFM responsiveness on Day 13 or Day 15 by NT-treated ewes was not different from that of roIFNτ-treated ewes. However, ewes which received either NT or roIFNτ had lower PGFM responses than ewes treated with SP.

Figure 4.1. Plasma PGFM of individual ewes which received intrauterine injections of SP (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP per uterine horn. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Days 13 and 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. PGFM profiles for ewe 15 (E15); ewe 35 (E35); and ewe 39 (E39) on Days 13 (circles) and Day 15 (squares) are shown in panels A, B and C, respectively.


A)
E15 D13
E15 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)


B)
E39 D13
E39 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

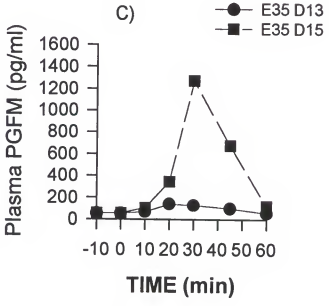
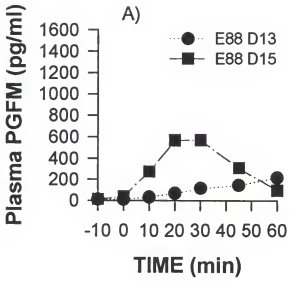
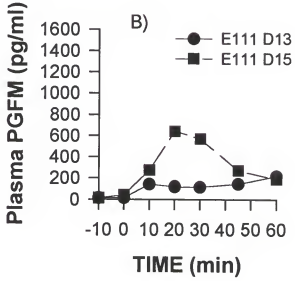

C)
E35 D13
E35 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

Figure 4.2. Plasma PGFM in individual ewes receiving intrauterine injections of NT (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 0.5 mg NT. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Days 13 and 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. PGFM profiles ewe 88 (E88); ewe 111 (E111); ewe 114 (E114) on Days 13 (circles) and Day 15 (squares) are presented in panels A, B, and C, respectively.


A)
E88 D13
E88 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)


B)
E111 D13
E111 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

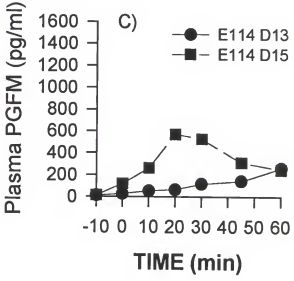
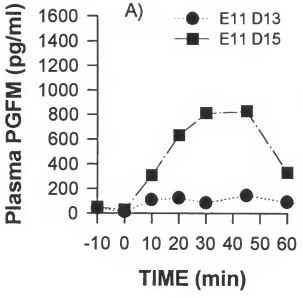
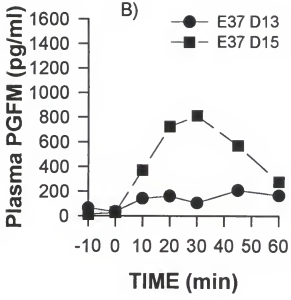
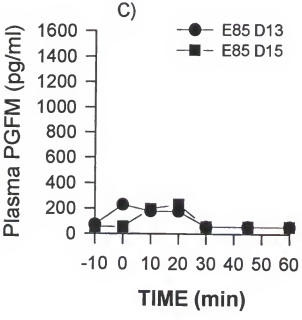

C)
E114 D13
E114 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

Figure 4.3. Plasma PGFM in individual ewes receiving intrauterine injections of roIFN$_\tau$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 50 μg roIFN$_\tau$ per uterine horn. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 13 and Day 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. PGFM profiles ewe 11 (E11); ewe 37 (E37); ewe 85 (E85); ewe 96 (E96) on Day 13 (circles) and Day 15 (squares) are presented in panels A, B, C, and D, respectively.


A)
E11 D13
E11 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)


B)
E37 D13
E37 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)


C)
E85 D13
E85 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

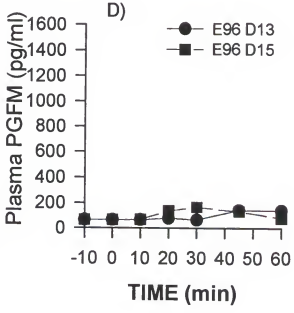

D)
E96 D13
E96 D15
Plasma PGFM (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
-10 0 10 20 30 40 50 60
TIME (min)

Figure 4.4. Mean plasma PGFM in ewes receiving intrauterine injections of SP, NT, or roIFN$_\tau$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT, or 50 μg roIFN$_\tau$ per uterine horn. The total protein concentrations per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 13 and Day 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. These values represent the mean main effects of treatment. Bars with the same letter are not different ($P>0.05$).

Discussion

In cyclic ewes, pulsatile release of $PGF_{2\alpha}$ from the endometrium between Days 14 and 15 of the estrous cycle causes luteolysis and ewes return to estrus. Establishment of pregnancy in the ewe requires abrogation of uterine release of luteolytic pulses $PGF_{2\alpha}$ to prevent luteolysis, maintain CL function and luteal cell production of progesterone (Roberts *et al.*, 1990; Bazer, 1991). During the period of pregnancy recognition in the ewe (Days 9-15; Moor and Rowson, 1966a, 1966b, 1966c; Farin *et al.*, 1990), the conceptus secretes oIFNτ (the pregnancy recognition factor in ewes). Ovine IFNτ inhibits development of the endometrial luteolytic mechanism and prevents pulsatile release of endometrial $PGF_{2\alpha}$ (Fintcher *et al.*, 1986; Vallet *et al.*, 1988; Ott *et al.*, 1992; Beard *et al.*, 1994; Beard and Lamming, 1994; Stevenson *et al.*, 1994; Spencer *et al.*, 1996; Spencer and Bazer, 1996). The cyclic increase of endometrial OTr on Day 14 in the ewe is blocked by intrauterine injection of oIFNτ (Mirando *et al.*, 1993; Spencer *et al.*, 1995, 1996; Spencer and Bazer, 1996). Progesterone, estrogen, and oxytocin, acting through their individual receptors mediate production of the pulsatile pattern of $PGF_{2\alpha}$ release by the endometrium (McCracken *et al.*, 1980, 1984; Silvia *et al.*, 1991; Wathes and Lamming,

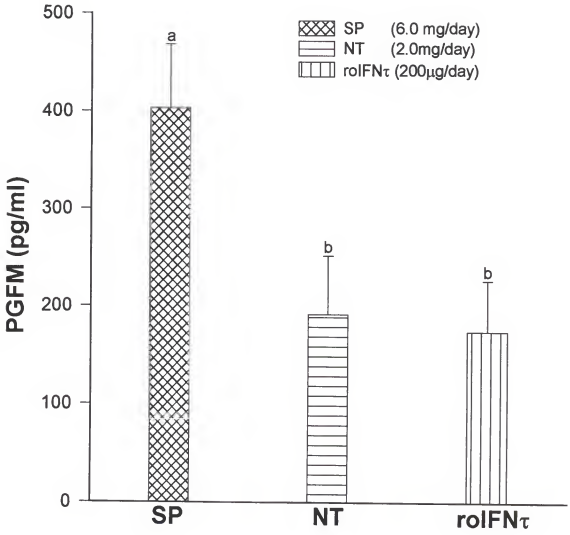

500
400
300
200
100
0
PGFM (pg/ml)
a
b
b
SP (6.0 mg/day)
NT (2.0mg/day)
rolFNτ (200μg/day)
SP
NT
rolFNτ

Figure 4.5. Mean plasma PGFM in ewes receiving intrauterine injections of SP, NT, or roIFN, (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT, or 50 μg roIFN, per uterine horn. The total protein concentrations per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 13 and Day 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. These values represent the mean main effects of day. Bars with the same letter are not different (P>0.05).

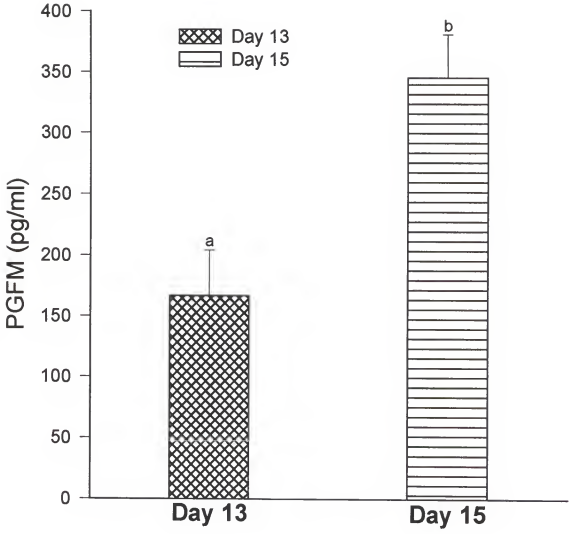

400
350
300
250
200
150
100
50
0
PGFM (pg/ml)
Day 13
Day 15
a
b
Day 13
Day 15

Figure 4.6. Mean plasma PGFM in ewes receiving intrauterine injections of SP, NT, or roIFN$_t$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT, or 50 μg roIFN$_t$ per uterine horn. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 13 and Day 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. These values represent the mean effects of the interaction of treatment by day. Bars with the same letter are not different ($P>0.05$).

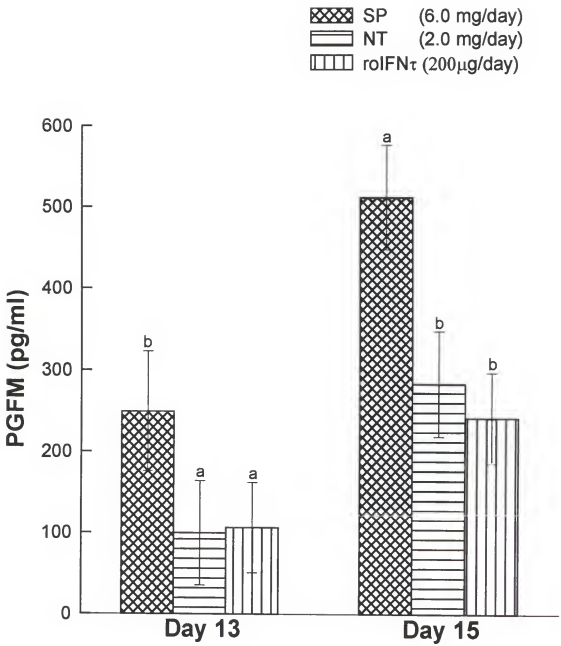
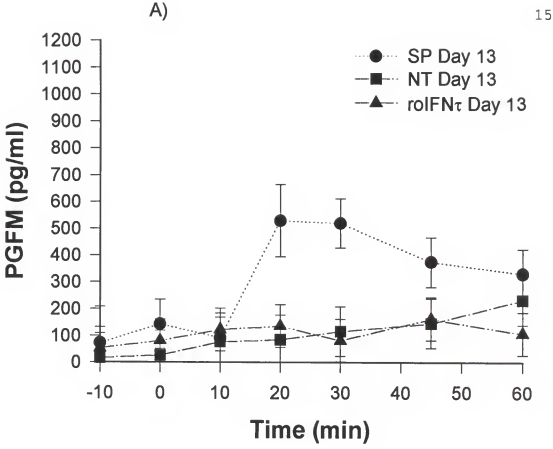
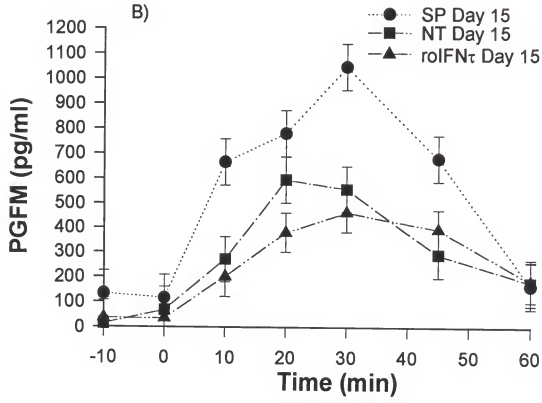

SP (6.0 mg/day)
NT (2.0 mg/day)
roIFNτ (200μg/day)
PGFM (pg/ml)
600
500
400
300
200
100
0
a
b
b
b
a
a
Day 13
Day 15

Figure 4.7. Mean plasma PGFM in ewes receiving intrauterine injections of SP, NT, or roIFN, (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP (circles), 0.5 mg NT (squares), or 50 μg roIFN, (triangles) per uterine horn. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 13 and Day 15 at time zero. Plasma samples were obtained 10 min prior to OT challenge, at time zero, and at 10, 20, 30, 45 and 60 min post-challenge. These values represent the interaction of treatment by day by time interactions for Day 13 and Day 15 are represented in panels A and B, respectively.


A)
SP Day 13
NT Day 13
roIFNτ Day 13
PGFM (pg/ml)
Time (min)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-10
0
10
20
30
40
50
60


B)
SP Day 15
NT Day 15
roIFNτ Day 15
PGFM (pg/ml)
Time (min)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-10
0
10
20
30
40
50
60

1995, Spencer *et al.*, 1996). In cyclic ewes, development of the endometrial luteolytic mechanism involves progesterone-induced down-regulation of Pr, which in turn allows up-regulation of Er and OTr on the endometrium. In pregnant ewes, Pr expression by endometrial epithelium is also down-regulated; however, up-regulation of Er and OTr is blocked by an as yet unknown effect of oIFNτ (Wathes and Hamon, 1993; Spencer *et al.*, 1995a; Spencer and Bazer, 1996). Through this mechanism the oxytocin-induced pulsatile release of $PGF_{2\alpha}$ by the endometrium is blocked. Results from the present study indicate that the NT peptide of oIFNτ is as effective as roIFNτ in attenuating the oxytocin-induced PGFM response in ewes.

CHAPTER 5
THE EFFECTS OF OVINE CONCEPTUS SECRETORY PROTEINS, RECOMBINANT OVINE INTERFERON *TAU* AND SYNTHETIC PEPTIDES CORRESPONDING TO PORTIONS OF INTERFERON *TAU* ON ENDOMETRIAL CONCENTRATIONS OF ESTROGEN AND PROGESTERONE RECEPTOR PROTEIN AND mRNA

Introduction

Establishment of pregnancy in ewes requires that the conceptus, by Day 12 of pregnancy (Moor and Rowson, 1966a) sends a message (oIFNτ; Godkin *et al.*, 1982; Vallet *et al.*, 1988; see Spencer *et al.*, 1996) to prevent luteolysis. Luteolysis is induced in ruminants by pulsatile secretion of $PGF_{2\alpha}$ by endometrial tissues (Hooper *et al.*, 1986; McCracken *et al.*, 1981, 1991). The pulsatile secretion of $PGF_{2\alpha}$ is initiated by secretion of oxytocin from the posterior pituitary and is escalated by oxytocin of ovarian origin (Flint *et al.*, 1990). Oxytocin from the ovary and $PGF_{2\alpha}$ from the uterus act together in a positive feedback loop to generate the luteolytic pulses required for luteolysis (Flint and Sheldrick., 1986; Hooper *et al.*, 1986; see Silvia *et al.*, 1991; McCracken *et al.*, 1991). However, the ability of endometrium to secrete $PGF_{2\alpha}$ in response to oxytocin does not develop until Day 13 to 14 of the cycle (Roberts *et al.*, 1975; Roberts and McCracken, 1976; Fairclough *et al.*, 1984;

Silvia *et al.*, 1991). Changes in endometrial responsiveness to oxytocin are due to differences in OTr concentrations within the uterus during the estrous cycle (Sheldrick and Flint, 1985; Beard and Lamming, 1994; Spencer *et al.*, 1995a; Wathes and Lamming, 1995).

During early pregnancy the pulsatile pattern of $PGF_{2\alpha}$ secretion is attenuated (Thornburn *et al.*, 1973; Barcikowski *et al.*, 1974; Moore *et al.*, 1982; Hooper *et al.*, Zarco *et al.*, 1988a) by the disruption of the oxytocin/$PGF_{2\alpha}$ positive feedback loop. Attenuation of the loop in the pregnant ewe is due to a block in endometrial expression of Er and the subsequent formation of OTr seen in the cyclic ewe just prior to luteolysis (Fairclough *et al.*, 1984; McCracken *et al.*, 1984; Sheldrick and Flint, 1985; Mirando *et al.*, 1990a, 1990b; Beard and Lamming, 1994; Wathes and Lamming, 1995; see Spencer *et al.*, 1996)

Ovine IFNτ acts through its transduction signal to prevent the cyclic increase in endometrial Er and OTr. Within cyclic ewes Er is abundant in the endometrial luminal epithelium just before and after estrus. Receptor numbers fall dramatically from Day 3, are low from Days 10 to 14, increase dramatically beginning on Day 14, and peak at estrus (Koligian and Stormshak, 1976; Miller *et al.*, 1977; Zelinski *et al.*, 1980; Cherny *et al.*, 1991; Ott *et al.*, 1993b). Cherny *et al.* (1991) reported that endometrial Er expression under steroidogenic control is not homogenous

throughout endometrial tissue. However, throughout these tissues, estrogen has been reported to be stimulatory to it's own receptor formation (Anderson *et al.*, 1975; Bhaloo and Katzenellenbogen, 1977; Zelinski *et al.*, 1980; Cherny *et al.*, 1991). Progesterone, on the other hand, is inhibitory to Er formation in several species (Brenner *et al.*, 1974; Hsueh *et al.*, 1975, 1976; Tseng and Gurpide, 1975; West *et al.*, 1976; Bhakoo and Katzenellenbogen, 1977) including sheep (Koligian and Stormshak, 1977b; Zelinski *et al.*, 1980; Cherny *et al.*, 1991). Control of expression of Er in the cyclic ewe may involve several factors (Cherny *et al.*, 1991) but in the pregnant ewe control of the Er is controlled by oIFNτ (Spencer *et al.*, 1995a, 1995b; Spencer and Bazer, 1996).

Progesterone in the cyclic animal is inhibitory to it's own endometrial receptor, through down-regulation mechanisms (Milgrom *et al.*, 1973; Leavitt *et al.*, 1974; Vu Hai *et al.*, 1977). In the cyclic, ewe progesterone's down-regulation of its own receptor in luminal epithelial and superficial glandular endometrium initiates removal of the proposed progesterone block (McCracken *et al.*, 1984), allowing Er up-regulation, and formation of the OTr, which when bound by oxytocin, initiates pulsatile $PGF_{2\alpha}$ production and luteolysis (Wathes and Hamon, 1993; Beard and Lamming, 1994; Spencer and Bazer, 1995, 1996; Wathes and Lamming, 1995; Spencer *et al.*, 1995a, 1995b, 1996, 1996). In pregnant

ewes, the Pr is also down-regulated by the extended period of elevated progesterone during diestrus (Ogle *et al.*, 1989, 1990; Ott *et al.*, 1993b; Mirando *et al.*, 1993; Wathes and Lamming, 1993; Spencer and Bazer, 1995) while at the same time the Er gene is prevented from the cyclic up-regulation of the Er, by removal of the progesterone-block, through the actions of oIFNτ (Wathes and Hamon, 1993; Spencer *et al.*, 1995a, 1995b; Spencer and Bazer, 1996). This in turn prevents the formation of endometrial OTr and, therefore, release of luteolytic pulses of $PGF_{2\alpha}$ in response to oxytocin by endometrium (see Wathes and Lamming, 1995; Spencer *et al.*, 1996). It is not known how oIFNτ blocks Er up-regulation but it is believed that oIFNτ signals the activation of transcription suppressors to block Er formation (see Spencer *et al.*,1996). Also unknown, is whether the entire oIFNτ molecule is required to elicit this resopnce, or if a portion of oIFNτ is able to induce the same response as the whole protein.

This study was undertaken to determine: (1) if roIFNτ is as effective as native oIFNτ in blocking endometrial Er and Pr expression; and (2) if oIFNτ, NT and CT are equally effective in blocking expression of Er and Pr.

In particular these experiments were to determine what effect treatment of cyclic ewes, with various synthetically produced peptides corresponding to overlapping segments of oIFNτ, had on endometrial Er mRNA, Er and Pr concentration.

The NT and CT peptides were first examined (Experiment 2; Er mRNA concentration only was examined for this experiment) due to the fact that they were the most extensively examined of the peptides (Pontzer *et al.*, 1990, 1994). It has been proposed that the NT possesses those properties which makes oIFNτ different from other IFNαs, and that CT is the portion common to the IFNαs. Experiment 3 examined the effect of the remaining peptides (2-5) on endometrial concentration of Er mRNA. Experiments 4, also examined the effect of NT treatment on endometrial concentration of Er mRNA, Er and Pr, but this experiment was specifically designed to determine what effect oxytocin challenge (*in vivo*) on Days 13 and 15 would have with regards to the main treatment of NT or roIFNτ. The final experiment, Experiment 5, was designed to determine what effect NT has on endometrial concentration of Er mRNA, Er and Pr through Day 18, which is two days longer than oIFNτ had been examined. This was to determine if NT has the ability to hold OTr concentration at a low level for an extended period of time.

Materials and Methods

Animals

Ewes of primarily Rambouillet breeding were checked daily at 07:30 AM for 20 min with vasectomized males of St. Croix or mixed Rambouillet breeding. Ewes which had

exhibited at least two normal estrous cycles (16 to 17 days in length) were assigned to experimental groups. Ewes for Experiments 1, 2, and 3 were housed at the Physiology Unit, Department of Animal Science, University of Florida. Ewes for Experiments 4 and 5 were housed at the Sheep Research Center, Texas A&M University.

Protein And Peptide Preparation

Ovine conceptus secretory protein preparation

Ovine conceptus secretory proteins were produced and prepared as described in Chapter 3.

Recombinant ovine interferon *tau* preparation

Recombinant ovine interferon *tau* was produced and prepared as described in Chapter 3.

Serum protein preparation

Serum protein was produced and prepared as described in Chapter 3.

Synthetic peptide production

Synthetic peptides corresponding to the NT and CT of oIFNτ were produced and prepared as described in Chapter 3.

Experimental Design

See Chapter 3 for the experimental designs of Experiments 2, 3, 4, and 5.

Estrogen Receptor mRNA

Ribonucleic acid isolation

Endometrial tissues for mRNA analysis were stored in plastic bags at -80°C until used. Tissues storage time was approximately 1.5 years for Experiment 2, 6 months for Experiment 3 and 2 months for Experiments 4 and 5. During this period of time homologous Er probes were developed for use. Ribonucleic acid was isolated by means of a single step guanididium thiocyanate-phenol-chloroform extraction method modified from the method described by Puissant and Houdebire (1990). Briefly, 1 gm of tissue was homogenized in 10 ml 4 M guanidine thiocyanate, 25 mM sodium citrate, sarkosyl 0.5% (w/v), pH 7.0, 0.1 M 2-mercaptoethanol at 4°C within a 50 ml conical tube. One ml of 2 M sodium acetate (pH 4.0), 10 ml of water-saturated phenol was added sequentially and gently mixed by inverting the tube after each addition. Two milliters of chloroform:isoamyl alcohol (49:1) was added and vortexed to mix. Tubes were centrifuged at 3,000 x g for 15 min. The upper phase was collected, and the RNA allowed to precipitate overnight at 20°C in 10 ml of isopropanol. Total RNA was pelletized by centrifugation at 3,000 g for 10 min, the supernate discarded, and the tubes inverted to drain. The pellet was resuspended in 2 ml LiCl (4 M) and total RNA repelletized by centrifugation at 3,000 x g for 15 min. The supernate was discarded and the tube inverted to drain. The pellet was

resuspended in 2 ml 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 0.5% SDS (w/v), and transferred to a 15 ml conical tube. Two milliters of chloroform was added, vortexed to mix and centrifuged at 3,000 x g for 10 min. The upper phase was collected, transferred to a sterile tube and 0.2 M sodium acetate added. Two milliters of isopropanol was added, and RNA allowed to precipitate overnight at -80°C. Total RNA was pelletized by centrifugation at 3,000 x g for 30 min. The supernate was discarded and the tubes with pelletized RNA inverted to drain. The pellet was washed with 1 ml 70% ethanol and recentrifuged at 3,000 x g for 10 min after which the tubes were inverted and allowed to dry for 15 min. Once dried, the pellet was suspended in 200 μl 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and transferred to a 1.5 microcentrifuge tube. A 5 μl aliquot was taken for quantification by spectrophotometric absorbance measurement at 260 nm. The remainder was stored in aliquots, at -80°C for northern blot and slot blot analyses.

Northern blot procedure

A 40 μl aliquot of each RNA sample was thawed and freeze-dried. The RNA was denatured in 15 μl 24 mM HEPES, 6 mM sodium acetate, 1.2 mM EDTA (pH 7.0), 50% formamide (v/v), and 2.2 M formaldehyde at 65°C for 15 min then rapidly chilled. Loading buffer (5 μl; 20 mM phosphate buffer, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added to samples just prior to gel loading. Samples

were electrophoresed through a 1.5% agarose, 2.2 M formaldehyde gel (12 h at 70 volts). Location of 18s and 28s ribosomal RNA bands as well as RNA integrity were determined by staining the gel in running buffer with 5 μl ethidium bromide (10 mg/ml). For northern bolt analysis, the RNA was transferred to a 0.45 μm nylon membrane (Biotrans, Irvine, CA) by capillary blotting. RNA was cross-linked to the filters by exposure to short-wave ultra-violet light for 2 min and then baked at 80°C for 2 h Membranes were stored in sealed hybridization bags until use. A northern blot was hybridized with each slot-blot as a control.

Slot blot procedure

Nylon membranes (Biotrans, Irvine CA) were prepared for RNA by soaking in water 5 min then in 10X SSC prior to being mounted on slot blot apparatus. After mounting each slot was rinsed with 500 μl 10X SSC. Total RNA (each sample in aliquots of 5, 10 and 20 μg RNA; yeast RNA was added to each blot as a control) was freeze dried, 125 μl denaturant (50% formamide, 6% formaldehyde, 20 mM Tris-HCl pH 7.5) was added and incubated at 65°C for 5 min. Twenty X SSC (125 μl) was added and the sample loaded on the membrane. Each slot was then rinsed with 500 μl 20X SSC. RNA was cross-linked to the filters by exposure to short-wave ultra-violet light for 2 min and then baked at 80°C for 2 h. Membranes were stored in sealed hybridization bags until use.

Isolation of ovine Er

The ovine Er cDNA probe (360 bp) was developed in our laboratory (Spencer *et al.*, 1993) and cloned using polymerase chain reaction with primers to the human Er mRNA sequence and reverse transcribed template from Day 16 cyclic ovine endometrial RNA. A clean probe was obtained by use of Geneclean (Bio 101 Inc, La Jolla, CA). Briefly, the linearized plasmid was run on a 1% TAE (Tris-HCl, acetate, EDTA) gel, the band excised, chopped into 2 mm^2 cubes and transferred to a 1.5 ml microcentrifuge tube. Impurities were removed from the DNA by the addition of sodium iodide (2.5 volumes of 6 M; supplied with Geneclean) to the gel and the addition of Glassmilk (silica matrix in water, supplied with Geneclean; 5 μl) to the gel mixture after melting at 55°C for 5 min. The Glassmilk was allowed to bind to the DNA for 5 min on ice and then pelletized by microcentrifugation for 5 sec to separate it into a clean fraction. The pellet was resuspended in 200 μl ice-cold "NEW" (NaCl, ethanol, water, supplied with Geneclean) three times and pelletized by centrifugation (5 sec). The pellet was resuspended in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and Glassmilk pelletized by microcentrifugation (30 sec) to yield the pure DNA in the supernatant. The final concentration of DNA after the Geneclean procedure was 0.172 $\mu g/\mu l$ as determined through quantification by spectrophotometric absorbance measurement at 280 nm.

The RNA probe was produced from the clean cDNA through the use of the Riboprobe Genmini System II kit (Promega Corp., Madison WI.) with T7 polymerase and labeled with ^{32}P (New England Nuclear, Boston, MA.; 800 Ci/mMol). Free radioactivity was separated from the specifically labeled probe by means of spin-quick chromatography columns (Select-D(RF); 5 Prime 3 Prime Inc., Bolder, CO.). A 1 μl aliquot of the labeled probe was counted to determine the radioactivity of the probe. Only probes with greater than 60 x 10^6 cpm were used for hybridization.

Hybridization

Northern blots and slot blots were pre-hybridized 2 h at 55°C in 20 ml hybridization buffer (50% formamide, 50 mM Na_2PO_4, 5X SSC, 0.1% SDS, 1.0 mM EDTA, 0.5X Denhardts, 200 μg/ml Herring sperm DNA). Pre-hybridization buffer was replaced with hybridization buffer (pre-hybridization buffer with radiolabled ovine Er probe; 20 X 10^6 dpm) and the membranes hybridized 19 h at 55°C. After hybridization the northern blot and slot blot membranes were washed sequentially, three times (20 min each wash; 68°C) in 0.1X SSC and 0.1% SDS, then three times (5 min each wash; room temperature) in 2X SSC. Hybridization signals were quantified using a Batascope 603 Blot Analyzer (Betagen) with the results given as CPM above background.

To correct for loading differences, membranes were stripped by boiling in 500 ml 0.5% SDS and 0.01X SSC for 20

min three times. The northern and slot blots were rinsed in 100 ml 0.1X SSC two times. The membranes were rehybridized to a human 28s rRNA cDNA and the signals quantified on blot analyzer. For each Er mRNA sample data, the corresponding 28s data was used as a covariate in analyses of covariance by SAS.

Estrogen Receptor Assay

Estrogen receptor concentration within endometrial tissues was determined in the laboratory of Dr. Tom Ogal (Medical College of Georgia). All receptor measurements were performed under conditions of endogenous steroid exchange which have been reported previously in sheep (Ott *et al.*, 1993b; Mirando *et al.*, 1993).

Endometrial tissues stored at -80°C (Experiments 4 and 5) were rapidly thawed and homogenized (ice-cold conditions were maintained throughout all procedures unless otherwise noted) by three 10 sec bursts of a tissumizer (Tekmar Co., Cincinnati, OH) followed by 30 sec periods of cooling in TEDSL (0.05M Tris-HCl (pH 7.8 at 4°C), 1.5 mM EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose and 0.2 mM leupeptin) buffer (5mg/ml). Centrifugation at 800 x g for 20 min resulted in a pellet fraction and a supernatant fraction. The supernatant was recentrifuged at 105,000 x g. The 800 x g pellet was washed three times by resuspension in TMDSL (0.05 M Tris-HCl (pH 7.8 at 4°C), 2.5 mM $MgCl_2$, 0.5 mM

dithiothreitol, 0.25 M sucrose and 0.2 mM leupeptin) buffer, followed by centrifugation at 800 x g for 20 min (each wash), and finally rehomogenization in TEDSL buffer using a Dounce homogenizer. A 0.25 ml aliquot of the supernatant fraction was incubated in a final volume of 0.3 ml in TEDSL.

Estradiol exchange was performed by incubating receptor preparations at 22°C for 2 h then 4°C for 18 h with incubation buffer containing six concentrations of ^{3}H-estradiol (0.3-3.0 nM; Dupont NEN Research, Boston, MA) Non-specific binding was determined in the presence of a 100-fold molar excess of unlabeled estradiol. Steroid-bound to the receptor was separated from free steroid by dextran-coated charcoal. Following charcoal extraction, free steroid was removed from the pellet fraction by three rinses with Tris-sucrose buffer, and finally suspended in 1 ml ethanol warmed to 30°C for 1 h. The suspension was re-pelletized by centrifugation at 1600 x g for 10 min and the supernate decanted into a scintillation vail for counting in a Beckman LS-1800 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Protein concentrations in homogenates were assayed by the method of Lowry *et al.* (1951), and DNA levels were determined using a fluorometric procedure (Hill and Whatley, 1975).

Progesterone Receptor Binding Assay

Progesterone receptor concentrations within endometrial tissues was determined in the laboratory of Dr. Tom Ogal (Medical College of Georgia). All receptor measurements were performed under conditions of endogenous steroid exchange which have been previously reported in sheep (Ott *et al.*, 1993b; Mirando *et al.*, 1993).

Homogenous aliquots of frozen (-80°C) endometrial tissues were rapidly thawed and mechanically homogenized at 0°C in TGDL buffer (0.01 M Tris-HCl, pH 7.8, 30% glycerol, 1 mM dithiothreitol and 0.2 mM leupeptin) by three 10 second bursts with a Tissumizer (Tekmar Co., Cincinnati OH) each followed by 30 seconds of cooling. Unless otherwise stated all procedures were carried out under ice-cold conditions. Following the final homogenization step the homogenate was filtered through two layers of gauze and centrifuged at 800 X g for 15 min. The supernatant was recentrifuged for 40 min at 157,000 X g. The receptors were purified partially by differential precipitation with ammonium sulphate, and 100 μl of partially purified cytosolic receptor were incubated with 0.2 ml TDGL for 20-22 h. Bound steroid was separated from free steroid by use of dextran-coated charcoal. The nuclear pellet was rinsed twice with fresh TDGL, suspended in TDGL buffer. Aliquots of 0.1 ml containing 0.1-0.2 mg DNA were incubated for 22 h at 4°C. Free steroid was removed from the pellet fraction by

repeated rinsing with TDGL and subsequently resuspended in 0.6 ml buffer.

Protein concentrations in homogenates were assayed by method of Lowery *et al.* (1951), and DNA levels were determined using a fluorometric procedure (Hill and Whatley, 1975).

The receptor exchange assay was performed by incubating receptor preparations with incubation buffer containing six concentrations of ^{3}H-labeled ligand (1.0-17 nM progesterone; Dupont NEN Research, Boston, MA). Nonspecific binding was determined in the presence of a 100 fold molar excess of unlabeled ligand. All receptor measurements were performed under conditions of endogenous steroid exchange (Ogle *et al.* 1989, 1990). Sample radioactivity was measured by liquid scintillation spectrometry (LS-1800, Beckman Instruments, Palo Alto, CA).

Statistical Analysis

Values for Er mRNA were measured from radiographs of slot blots in CPM above background. Values were corrected for loading variation prior to analysis by dividing each Er mRNA CPM by the corresponding 28s CPM value from the same striped membrane (Ott *et al.*, 1993b). The resulting relative unit value was subjected to analysis. Data were subjected to least squares means analysis of variance using the general linear models procedure of the Statistical Analysis

System (SAS Institute, 1995). Ewes were nested within treatment and all tests of hypothesis were performed using the appropriate error terms according to the expectation of the mean squares (Snedecor and Cochran, 1980). Data are expressed in least squares means ± S.E.M. Within status comparisons among days were made using orthogonal contrasts of the means.

Steroid hormone receptor assays were analyzed by computerized curve-fitting programs described previously by Ogle *et al.* (1989). Measurements of receptor binding were normalized for DNA and expressed as total receptor content. Data were subjected to least squares means analysis of variance using the general linear models procedure of the Statistical Analysis System (SAS Institute, 1995). Ewes were nested within treatment and all tests of hypothesis were performed using the appropriate error terms according to the expectation of the mean squares (Snedecor and Cochran, 1980). Data are expressed in least squares means ± S.E.M. Within status comparisons among days were made using orthogonal contrasts of the means.

Results

Estrogen Receptor mRNA

Endometrial tissues were collected for determination of Er mRNA concentration in Experiments 2, 3, 4, and 5. The results are shown in Figures 5.1 through 5.4, respectively. Although only one level of RNA is shown in the figures (5 μg RNA) there was a linear increase in CPM over the three levels of RNA loaded onto each membrane. All CPM values were corrected for loading variation with 28s CPM values from the striped membranes prior to analysis of the data.

In Experiment 2 there was no difference in endometrial Er mRNA concentration between SP and oCSP treatment (Fig. 5.1A). Treatment with no peptide (0 Pep), the NT peptide of oIFNτ or the C-terminus of oIFNτ also were not different in their effect on endometrial Er concentration (Fig. 5.1B) and there was no effect of the interaction of treatment by peptide (Fig. 5.1C).

The results for Experiment 3 are shown in Figure 5.2. There were no differences in endometrial Er mRNA concentration in tissues collected from ewes treated with SP or Peptides 1 through 5. Endometrial Er mRNA concentration was lower in tissues collected from ewes treated with roIFNτ as compared with SP-, Pep 2- and Pep 3-treated ewes.

Furthermore, Er mRNA in ewes treated with roIFNτ was not different from ewes treated with NT, Pep 4 and Pep 5.

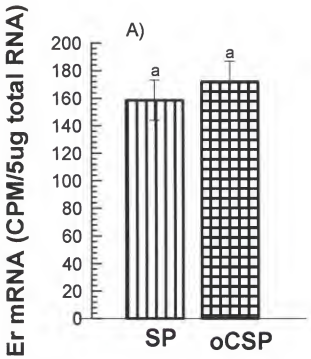
Oxytocin challenge as in Experiment 4 apparently did not alter the overall findings of Experiment 3 as they relate to endometrial Er mRNA concentration (Fig. 5.3). Inthis study endometrial Er mRNA concentration was lower in ewes treated with roIFNτ compared to SP-treated control ewes and ewes treated with NT. There were no differences in mRNA concentrations in endometrial tissues collected from ewes treated with NT and ewes treated with SP.

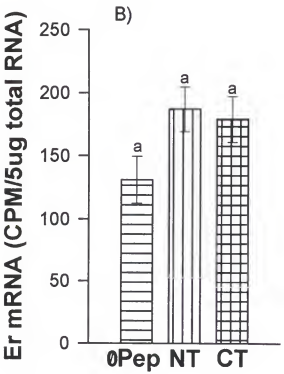
The results for Experiment 5 are shown in Fig. 5.4 A, B, and C. No difference was found in mRNA concentrations within endometrial tissues collected from ewes treated with SP compared with ewes treated with NT. There also was no change in Er mRNA concentration across the days examined (Day 16, 17 and 18) and there was no differences found after contrasting the means of the treatment*day interaction

Estrogen Receptor Binding Assay

Results from the analysis of endometrial Er binding evaluation indicating total endometrial Er concentration for Experiment 4 and Experiment 5 are shown in Fig. 5.5 and 5.6A, B, and C, respectively. Er binding was lower in ewes treated with roIFNτ or NT as compared with SP-treated animals in Experiment 4. There was no difference in the Er concentrations between ewes treated with NT and roIFNτ in

Figure 5.1. Endometrial Er mRNA in cyclic ewes receiving intrauterine injections of SP, oCSP, the peptides CT and NT, or combinations of the proteins and peptides (Experiment 2). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 mg NT + 1.0 mg SP, 0.5 mg CT + 1.0 mg SP, 0.75 mg oCSP + 0.75 mg SP, 0.75 mg oCSP + 0.5 mg NT or 0.75 mg oCSP + 0.5 mg CT. Treatments began on Day 12 and continued through the morning of Day 16. Main effects of treatment, peptide treatment, and the interaction of protein by peptide are represented in panels A, B and C, respectively. Zero (0) Pep indicates ewes were treated with the main treatment of SP or oCSP only. Bars with the same letters are not different ($P>0.05$).


A)
Er mRNA (CPM/5ug total RNA)
200
180
160
140
120
100
80
60
40
20
0
a
a
SP
oCSP


B)
Er mRNA (CPM/5ug total RNA)
250
200
150
100
50
0
a
a
a
0Pep NT CT

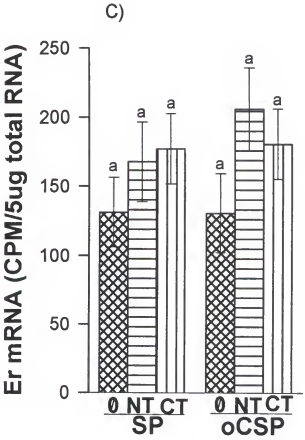

C)
Er mRNA (CPM/5ug total RNA)
250
200
150
100
50
0
a
a
a
a
a
a
0 NT CT
SP
0 NT CT
oCSP

Figure 5.2. Endometrial Er mRNA in ewes receiving intrauterine injections of SP, synthetically produced peptides of oIFN$_t$, or roIFN$_t$ (Experiment 3). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.25 μg roIFN$_t$, or 0.5 mg each of synthetic peptide NT, 2, 3, 4, or 5. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 12 and continued through the morning of Day 16. Bars with the same letters are not different ($P>0.05$).

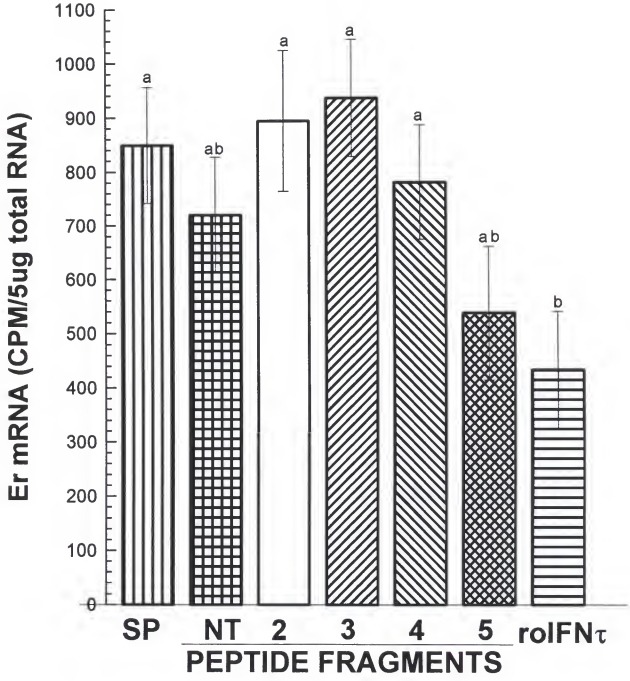
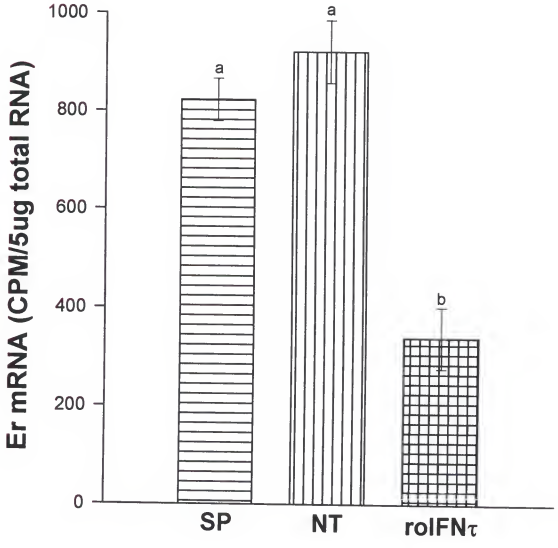

Er mRNA (CPM/5ug total RNA)
1100
1000
900
800
700
600
500
400
300
200
100
0
a
ab
a
a
a
ab
b
SP
NT
2
3
4
5
roIFNτ
PEPTIDE FRAGMENTS

Figure 5.3. Endometrial Er mRNA in ewes receiving intrauterine injections of SP, NT, or roIFN$_t$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 μg roIFN$_t$ or 0.5 mg NT. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of oxytocin (10 i.u.) on Day 14. Bars with the same letters are not different ($P>0.05$).

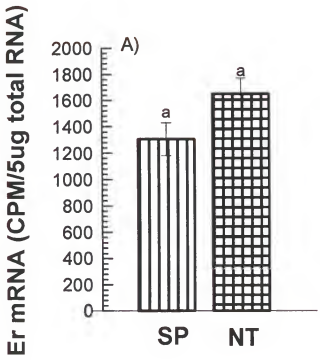

Er mRNA (CPM/5ug total RNA)
1000
800
600
400
200
0
a
a
b
SP
NT
roIFNτ

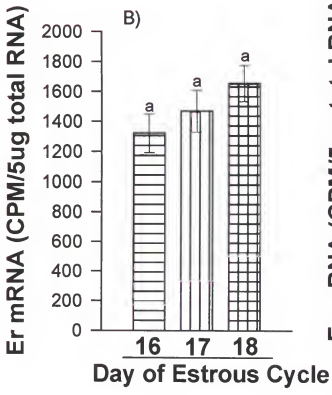
this study (Fig. 5.5) In Experiment 5, endometrial Er concentration also was lower in ewes treated with NT as compared with SP-treated ewes (Fig. 5.6A). Endometrial Er concentration increased over the days examined (Day 16, 17 and 18) with Er concentration being greater in tissues collected from ewes on Day 18 than in tissues collected from ewes on Day 16 or 17 (Fig. 5.6B). This increase in endometrial Er concentration was due to an increase in the Er numbers in tissues collected from ewes treated with SP from Day 16 to Day 18. There was no increase in Er numbers over the days examined in tissues collected from ewes treated with NT (Fig. 5.6C). The NT was effective in blocking the rise in endometrial Er concentration seen in ewes treated with SP.

Progesterone Receptor Binding Assay

Results for the analysis of Pr binding determination indicating total receptor concentration within endometrial tissues examined in Experiment 4 and Experiment 5 are shown in Figures 5.7 and 5.8, respectively. As expected, due to down-regulation of the Pr prior to Day 16, there was no difference in Pr concentration within endometrial tissues collected from ewes treated with SP, NT or roIFNτ in Experiment 4 (Fig. 5.7). In Experiment 5 there was also no difference in receptor concentration between treatments (Fig. 5.8A) or between days on which tissues were collected

Figure 5.4. Endometrial Er mRNA in ewes receiving intrauterine injections of SP or NT (Experiment 5). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP or 0.5 mg NT + 1.0 mg SP. Treatments began on Day 11 and continued through the morning of Day 16, 17 or 18 when tissues were collected. Main effects of treatment, main effects of day and the effects of the interaction of treatment by day are presented in panels A, B, and C, respectively. Bars with the same letters are not different (P>0.05).


A)
Er mRNA (CPM/5ug total RNA)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
SP
NT


B)
Er mRNA (CPM/5ug total RNA)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
a
16
17
18
Day of Estrous Cycle

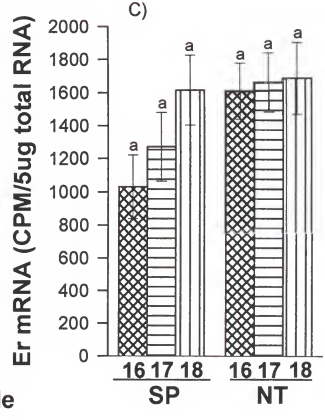

C)
Er mRNA (CPM/5ug total RNA)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
a
a
a
a
16 17 18
SP
16 17 18
NT

Figure 5.5. Total endometrial Er in ewes receiving intrauterine injections of SP, NT, or roIFN$_t$ (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 μg roIFN$_t$ or 0.5 mg NT. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of oxytocin (10 i.u.) on Day 14. Bars with the same letter are not different ($P>0.05$).

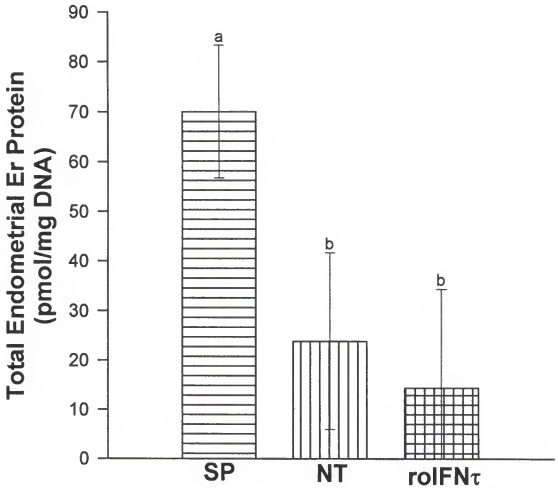
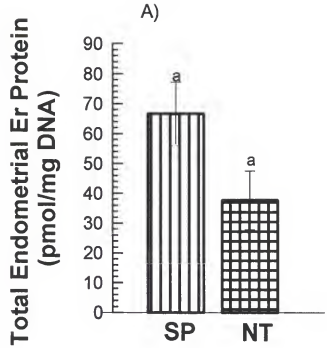
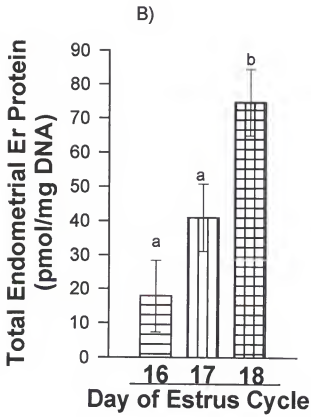
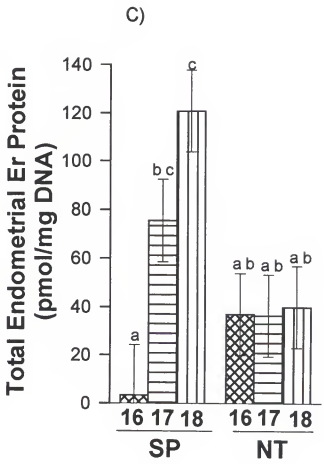

Total Endometrial Er Protein
(pmol/mg DNA)
90
80
70
60
50
40
30
20
10
0
a
b
b
SP
NT
roIFNτ

Figure 5.6. Total endometrial Er in ewes receiving intrauterine injections of SP or NT (Experiment 5). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP or 0.5 mg NT + 1.0 mg SP. Treatments began on Day 11 and continued through the morning of Day 16, 17 or 18 when tissues were collected. Main effects of treatment, effects of day and effects of the interaction of treatment by day are represented in panels A, B and C, respectively. Bars with the same letters are not different (P>0.05).


A)
Total Endometrial Er Protein (pmol/mg DNA)
90
80
70
60
50
40
30
20
10
0
a
a
SP
NT


B)
Total Endometrial Er Protein (pmol/mg DNA)
90
80
70
60
50
40
30
20
10
0
a
a
b
16 17 18
Day of Estrus Cycle


C)
Total Endometrial Er Protein (pmol/mg DNA)
140
120
100
80
60
40
20
0
a
b c
c
a b
a b
a b
16 17 18
16 17 18
SP
NT

regardless of treatment (Fig. 5.8B). Contrasts of the means of the treatment*day interaction revealed that the receptor concentration on Day 16 from ewes treated with NT was greater than from ewes treated with SP, regardless of the day examined, and also was greater than the receptor concentration found within tissues of ewes treated with NT that were collected on Day 18. While there was no change in the receptor concentration within endometrial tissues collected from ewes treated with SP across the days examined the Pr numbers fell dramatically within tissues from ewes treated with NT between Day 16 and Day 18.

Discussion

In ewes, pulsatile production of $PGF_{2\alpha}$ by the endometrium is primarily controlled by the steroid hormones progesterone and estrogen, which act in concert to regulate OTr formation (Soloff, 1975; McCracken, 1984; Sheldrick and Flint, 1985; Meyer *et al.*, 1988; Zang *et al.*, 1992; Beard *et al.*, 1994; Spencer and Bazer, 1995). McKracken *et al.* (1984) was the first to suggest that progesterone acts to block OTr formation.

Until recently our working hypothesis had been that oIFNτ stabilized or up-regulated the endometrial Pr population to prolong the progesterone block (McCracken, 1984) to Er formation and the subsequent OTr formation (Ott

Figure 5.7. Total endometrial Pr in ewes receiving intrauterine injections of SP, NT, or roIFN, (Experiment 4). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP, 0.5 μg roIFN, or 0.5 mg NT. The total protein concentration per treatment were balanced to 1.5 mg with SP. Treatments began on Day 11 and continued through the morning of Day 16. All ewes were challenged with a single injection of OT (10 i.u.) on Day 14. Bars with the same letters are not different (P>0.05).

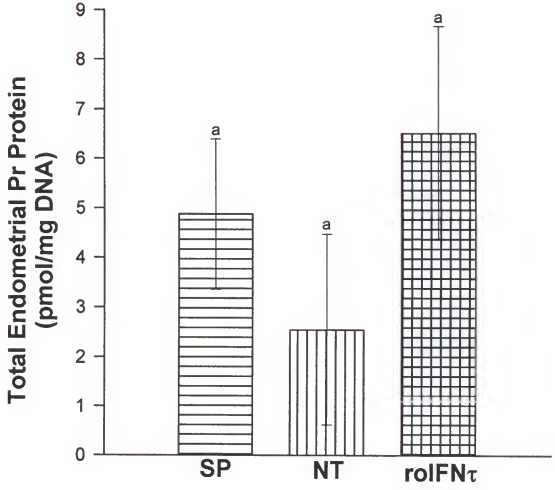
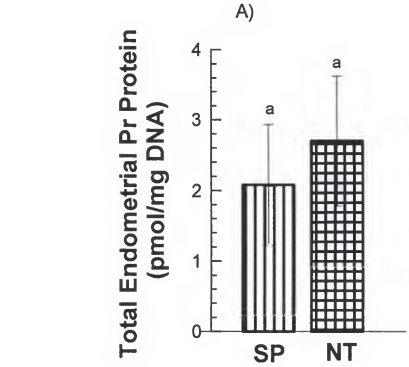
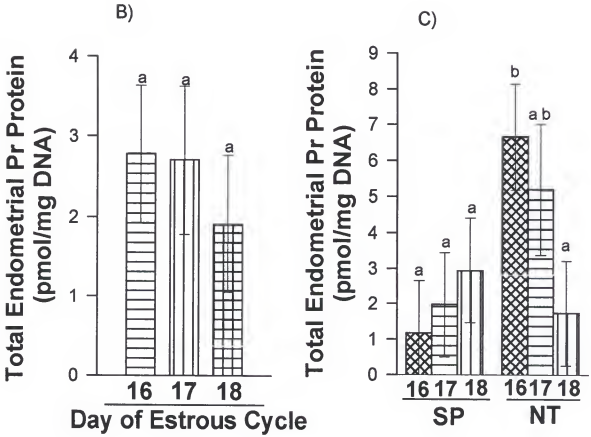

Total Endometrial Pr Protein
(pmol/mg DNA)
9
8
7
6
5
4
3
2
1
0
a
a
a
SP
NT
roIFNτ

Figure 5.8. Total endometrial Pr in ewes receiving intrauterine injections of SP or NT (Experiment 5). Each ewe received twice daily intrauterine injections (06:00 and 18:00 h, respectively) of 1.5 mg SP or 0.5 mg NT + 1.0 mg SP. Treatments began on Day 11 and continued through the morning of Day 16, 17 or 18 when tissues were collected. Main effects of treatment, effects of day, and effects of the interaction of treatment by day are represented in panels A, B and C, respectively. Bars with the same letters are not different (P>0.05).


A)
Total Endometrial Pr Protein (pmol/mg DNA)
4
3
2
1
0
a
a
SP
NT


B)
Total Endometrial Pr Protein (pmol/mg DNA)
4
3
2
1
0
a
a
a
16 17 18
Day of Estrous Cycle
C)
Total Endometrial Pr Protein (pmol/mg DNA)
9
8
7
6
5
4
3
2
1
0
a
a
a
b
a b
a
16 17 18
SP
16 17 18
NT

et al., 1993; Mirando *et al.*, 1993). However, the current hypothesis is oIFNτ does not stabilize or up-regulate Pr, but that the actions of oIFNτ are to block Er gene expression and subsequent increase in endometrial OTr concentration which allows for the luteolytic release of $PGF_{2\alpha}$ (Spencer and Bazer, 1995; Spencer *et al.*, 1996).

How pregnant ewes prevent up-regulation of endometrial Er is not fully understood. Clearly the developing embryonic secretory product, oIFNτ, prevents development of the luteolytic mechanism and pulsatile release of $PGF_{2\alpha}$ (Vallet *et al.*, 1988). In pregnant ewes the increased concentration of the Er (Findlay *et al.*, 1982; Mirando *et al.*, 1993; Ott *et al.*, 1993) and OTr (Roberts *et al.*, 1976; Ott *et al.*, 1993) are not seen as observed in the cyclic ewe after 10 days of progesterone exposure. Intrauterine injection of oCSP inhibits oxytocin-induced endometrial phosphoinositol hydrolysis (Mirando *et al.* 1990; Ott *et al.*, 1992; Chapter 3) and $PGF_{2\alpha}$ secretion (Vallet *et al.*, 1990; Mirando *et al.*, 1990; Chapter 4). While Mirando *et al.*, (1993) reported that oCSP intrauterine injection prevented increases in Er mRNA and protein in cyclic ewes, no such block was found in Experiments 2. In Experiment 2 the endometrial concentration of Er mRNA was the same for SP-treated ewes as it was for oCSP-treated ewes. However, in Experiments 4 and 5, intrauterine injection of roIFNτ did block the increase in Er mRNA as noted by others for cyclic

ewes. The difference between these two current studies could be attributed to the days in which the intrauterine injections were administered, in the case of the oCSP the injections were given from Day 12 to Day 16 while in the roIFNτ study the injections were administered from Day 11 to Day 16. Since Day 12 has been identified as the "critical" day for maternal recognition to occur (Moor and Rowson, 1964; 1966a) it could be that the ewes receiving oCSP were on the boarder line of this critical period and were not as receptive to the effects of the oCSP. Alternatively, these results could be attributed to the fact that these samples were stored at -80°C for a prolonged period, while homologous probes were being developed, and this could have affected the stability of the mRNA.

Interestingly, NT decreased Er protein without affecting levels of Er mRNA or gene expression. This could be attributed to either destabilization of the transcripts as well as other post-transcriptional effects on the mRNA, or prevention of translation. The RNA could be affected by the activation of 2'-5' A synthetase, which activates endonucleases that degrade RNA. Translation could be adversely effected by the actions of IFN to inactivate the translation initiation factor eIF-2α (Singer and Berg, 1991). All of these actions could be explained by partial agonist activity of NT. The question then becomes, how is this partial agonist signal by NT, at least in regards to Er

mRNA, seen by the endometrium as different from that of roIFNτ. There is no data to convincingly answer this question, but it would seem plausible that the NT, in binding to the Type-I IFN receptor initiates a conformational change in the receptor, or at least some change in receptor signal, that would account for these differences.

Collectively, these results support our current working hypothesis that oIFNτ prevents the cyclic increase in endometrial Er but this effect is not through an effect of roIFNτ to stabilize or up-regulate Pr. Also, all instances examined in the present study, except for the case of the Er mRNA, the NT acted the same as did the complete roIFNτ molecule indicating that the specific maternal recognition properties of oIFNτ reside within the amino-terminal portion of this molecule.

CHAPTER 6
GENERAL DISCUSSION

Working Model of Maternal Recognition of Pregnancy in the Ewe

A working model for the events involved in the maternal recognition of pregnancy in the ewe has been developed based on the findings of Spencer and others (Bazer, 1992; Spencer and Bazer, 1996; Spencer *et al.*, 1995a, 1995b; Bazer *et al.*, 1995). According to this model, it has been proposed that for cycling ewes, Er are present in the occupied state on uterine epithelium during metestrus, suggesting that OTr are present in this tissue as well. Pr are also present, but insufficient numbers of Pr are occupied to suppress synthesis of OTr due to low circulating levels of progesterone. During diestrus, endometrial Er and estradiol in circulation are low and occupied Pr initiate and maintain the progesterone block to synthesis of Er and OTr for approximately 10-12 days. During late diestrus, progesterone down-regulates Pr which allows up-regulation of Er and OTr which is accompanied by increasing secretion of estradiol by the ovarian follicles. The pulsatile release

of oxytocin from the CL and posterior pituitary initiates the release of luteolytic pulses of prostaglandin from the endometrium to destroy the CL.

In the cyclic ewe during late diestrus (Fig. 6.1), Pr is undetectable in the luminal and superficial glandular epithelium. In the absence of suppression by progesterone, Er gene transcription increases in the epithelium which allows for Er-mediated increases in OTr formation. When pregnancy occurs (Fig. 6.2), the conceptus produces antiluteolytic signal(s) between Days 10 and 21 which acts on the endometrial steroid hormone receptors during pregnancy recognition to inhibit the formation of OTr and the subsequent pulsatile release of luteolytic prostaglandins. This luteolytic signal in the ewe has been demonstrated as being oIFNτ, which acts to prevent OTr formation in the Pr-negative luminal and superficial glandular epithelium. Ovine IFNτ attenuation of OTr prevents pulsatile production of luteolytic $PGF_{2\alpha}$, this in turn maintains CL production of progesterone. Continued progesterone production in turn acts on the Pr-positive stroma and deep glandular epithelium to suppress estrogen-induced increases in Er and OTr gene expression in these tissues. These actions prevent the pulsatile production of $PGF_{2\alpha}$ by cells of the stroma and deep glandular epithelium. The mechanisms by which oIFNτ acts (Fig. 6.3) are presumed to be mediated through the binding to Type-I INF receptors

Figure 6.1: Schematic diagram depicting a working model for the events involved in regulation of hormone receptor expression in the luminal and shallow glandular epithelium (Top) and stroma and deep glandular epithelium (Bottom) during late diestrus in the cyclic ewe. During this time, circulating levels of progesterone are low while estradiol concentrations increase. Pr is undetectable in the luminal and shallow glandular epithelium, and in the absence of suppression by progesterone, Er gene transcription increases in the epithelium allowing for Er-mediated increases in Otr formation, and the subsequent increase in the pulsatile release of $PGF_{2\alpha}$.

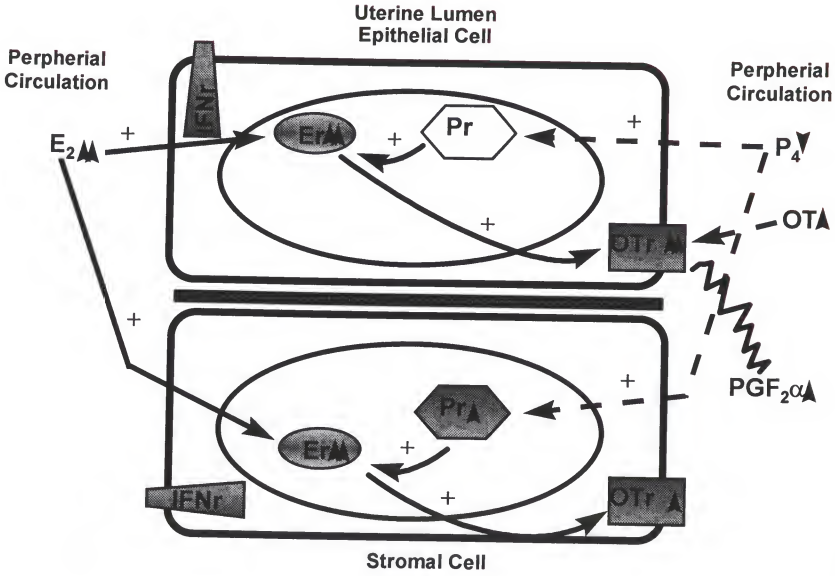

Uterine Lumen
Epithelial Cell
Perpherial
Circulation
Perpherial
Circulation
ENr
E2
ErA
Pr
P4
OT
OTr A
PrA
PGF2α
ErA
IFNr
OTr A
Stromal Cell

Figure 6.2: Schematic diagram depicting a working model for the events involved in regulation of hormone receptor expression in the luminal and shallow glandular epithelium (Top) and stroma and deep glandular epithelium (Bottom) during pregnancy recognition in the ewe. During this time, the conceptus produces antiluteolytic signals (oIFN$_t$) which acts to suppress transcription of the Er gene in the Pr-negative luminal and shallow glandular epithelium, leading to the prevention of OTr formation, inhibition of luteolytic PGF$_2\alpha$ release, and maintenance of progesterone production. Progesterone then acts on the stroma and deep glandular epithelium to suppress Er and OTr expression through as yet unknown mechanisms. The combined efforts of oIFN$_t$ and progesterone thus leads to the blockage of OTr formation in the entire endometrium.

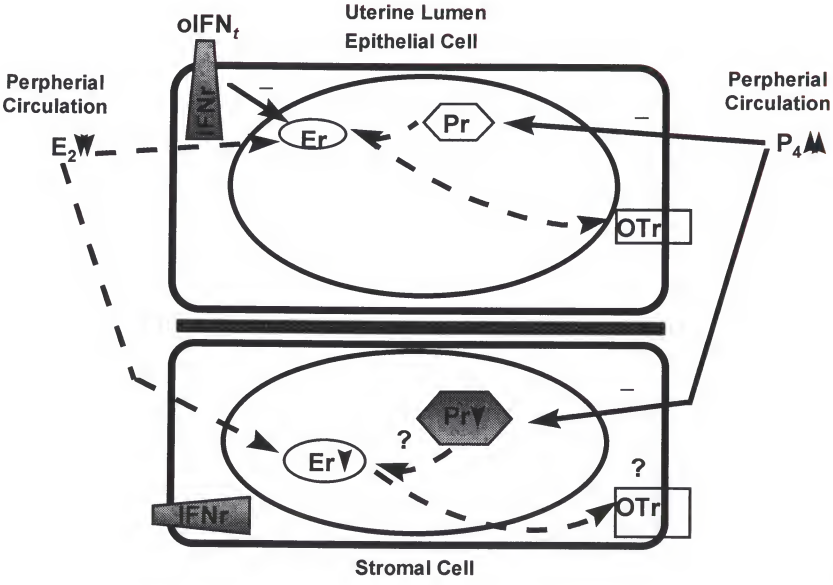
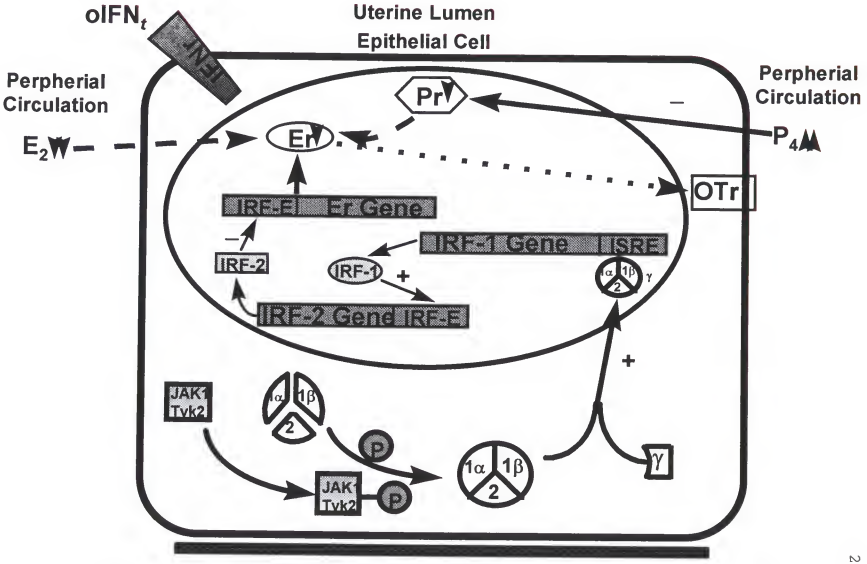

oIFN$_t$
Uterine Lumen
Epithelial Cell
Perpherial Circulation
Perpherial Circulation
IFNr
–
Er
Pr
–
E$_2$
P$_4$
OTr
–
Pr
?
Er
?
OTr
IFNr
Stromal Cell

Figure 6.3: Schematic diagram depicting a working model for the events involved in oIFN, regulation of hormone receptor expression in the endometrial epithelium during pregnancy recognition in the ewe. oIFN, binds to receptors present on the luminal and superficial glandular epithelium of the endometrium and causes phosphorylation (activation) of two cytoplasmic tyrosine kinases, Jak1 and Tyk2, which subsequently cause the activation of STAT1α, STAT1ß and STAT2 and the association of this complex, with ISGF3 gamma. The ISGF3 transcription complex then translocates to the nucleus where they bind to specific ISRE on the gene for IRF-1. The IRF-1 gene is transcribed and then translated, and the resulting IRF-1, a positively-acting transcription factor, binds to the IRF-E of the IRF-2 gene. This gene is transcribed and translated, and the resulting negatively-acting transcription factor binds to the IRF-E of the Er gene and inhibits transcription of the message for Er. Reduction in Er gene expression leads to the prevention of OTr formation, inhibition of luteolytic $PGF_2\alpha$ release and, therefore, maintenance of progesterone production, and successful establishment of pregnancy.


oIFNt
IFNR
Uterine Lumen
Epithelial Cell
Perpherial Circulation
Perpherial Circulation
E2
Er
Pr
P4
OTr
IRF-E
Er Gene
IRF-2
IRF-1 Gene
ISRE
IRF-1
IRF-2 Gene
IRF-E
JAK1
Tyk2
1α
1β
2
γ
P

present on the luminal and superficial glandular epithelium of the endometrium. Changes in confirmation of the IFN receptor activate two cytoplasmic tyrosine kinases, Jak1 and Tyk2. These activate through phosphorylation, proteins within the cytoplasm which comprise the interferon stimulated gene factor-3 complex. The interferon stimulated gene factor-3 complex, subsequently translocates to the nucleus. It has been proposed that translocation of the signal into the nucleus requires a sequence which facilitates that translocation of the signal transduction complex, interferon stimulated gene factor-3. There is a nuclear translocation sequence located on the CT of the full length oINFτ molecule. There may also be other nuclear translocation sequences located on the cellular portion of the Type-I IFN receptor, on various components of the JAK/STAT signal transduction pathway, or on, as yet unknown, components of the signal cascade.

Regardless of where the nuclear translocation sequence is located, once the signal has made its way into the nucleus, it binds to specific interferon stimulated response elements on DNA which direct IFN-induced transcriptional responses.One of these responses is the ultimate transcription of interferon regulatory factor-1 and interferon regulatory factor-2, transcription factors that act either positively or negatively, respectively. It has been proposed that oIFNτ causes an increase in the

interferon regulatory factor-2 to interferon regulatory factor-1 ratio, which may then suppress the expression of Er and Otr.

Thus, the combined actions of oIFNτ and progesterone result in suppression of OTr formation in the entire endometrium, reduction in pulsatile secretion of PGF, maintenance of the CL, and the successful establishment of pregnancy. This model has been used as to generate a working hypothesis that has been tested herein. Specifically, studies were designed to determine the function of oIFNτ on factors associated with maternal recognition and to examine the functional properties of specific domains of oIFNτ responsible for maternal recognition of pregnancy effects.

Effects of oIFNτ on IP metabolism and OTr Concentrations

Our working hypothesis predicts that roIFNτ or NT, working through the signal transduction pathway for oINFτ, would decrease pulsatile $PGF_{2\alpha}$ secretion. We have shown that IP metabolism is directly associated with the mechanism of OTr action. IP metabolism is attenuated by the actions of oCSP, roIFNτ and NT on endometrial tissues. Furthermore, endometrial OTr concentration is also reduced by these treatments. Collectively these results support the

hypothesis that oIFNτ blocks OTr formation leading to prevention of the luteolytic release of $PGF_{2\alpha}$.

Effects of oIFNτ on Oxytocin-Stimulated PGFM

According to our working hypothesis pregnancy recognition ultimately requires control of pulsatile prostaglandin secretion by the uterine epithelium which is controlled by oxytocin through OTr. We have demonstrated, by measuring PGFM in plasma, that roIFNτ reduces pulsatile release of $PGF_{2\alpha}$. Similar results were obtained following treatment of ewes with NT. In addition, both roIFNτ and NT had the same affect on plasma PGFM as did oCSP in previous studies from this laboratory. This further supports our hypothesis that there is a direct link between oIFNτ and the control of $PGF_{2\alpha}$.

Effects of oIFNτ on Er Protein and mRNA

The current model for maternal pregnancy recognition signaling by oIFNτ predicts that modulation Er expression plays a fundamental role in the mechanisms by which oIFNτ prevents OTr expression and ultimately pulsatile $PGF_{2\alpha}$ secretion. Results from these studies have confirmed that in ewes roIFNτ decreases both the message and the protein for Er, but does not affect Pr. This is the first report that NT decreased Er protein without affecting Er mRNA.

The fact that NT decreased Er protein without affecting levels of Er mRNA or gene expression, brings about an interesting question. How does NT induce endometrial cells to act differently than roIFNτ in regards to Er mRNA and identical to roIFNτ as it relates to Er. This could be attributed to either destabilization of the transcripts as well as other post-transcriptional effects on the mRNA, or prevention of translation. The RNA could be affected by the activation of 2'-5' A synthetase, which activates endonucleases to degrade RNA. Translation could be adversely effected by the actions of IFN to inactivate the translation initiation factor elF-2α (Singer and Berg, 1991). All of these actions could be explained by partial agonist activity of NT. The question then becomes, how is this partial agonist signal by NT, at least in regards to Er mRNA, seen by the endometrium as different from that of roIFNτ. There is no data with which to answer this question, but it could be possible that the NT, in binding to the Type-I IFN receptor initiates a conformational change in the receptor, or at least some change in receptor signal, that would account for these differences. In light of the fact that NT has no nuclear translocation signal (it is found on the CT of the full length oINFτ molecule) these results also indicate that there is an additional signal somewhere within the pathway. It could be located on the cytoplasmic side of the Type-1 IFN receptor, one of the

components of the JAK/STAT signal transduction pathway, or a component of the pathway yet to be found. In any case, the differences between the action of NT and oIFNτ warrants further study.

Summary

During the process of maternal pregnancy recognition in the ewe, control of $PGF_{2\alpha}$ release is critical to the maintenance of pregnancy. As discussed earlier, oIFNτ accomplishes this by preventing Er expression in luminal epithelium and superficial glandular endometrial tissues. By controlling Er expression in these cells oIFNτ's actions set into motion a series of events which not only prevents the luteolytic release of $PGF_{2\alpha}$, but also maintains production of progesterone by the CL. Continued production of progesterone by the CL is also important in the stromal and deep glandular endometrium to bind Pr and prevent expression of Er and OTr in these tissues, completing the block to pulsatile $PGF_{2\alpha}$ release.

While the experiments discussed in this dissertation were not designed to address the temporal and spatial effects of oIFNτ, they support the current hypothesis which delineates the mechanism of action of oIFNτ as the maternal pregnancy recognition factor in the ewe. Specifically, in endometrial tissues roIFNτ and NT decrease Er expression

protein, thereby preventing up-regulation of OTr, which in turn prevents oxytocin-induced pulsatile secretion of $PGF_{2\alpha}$.

APPENDIX A
PROTOCOL FOR PGFM ASSAY

Standards (triplicates)

1. Pipette 200 μl Banamine-treated plasma into standard tubes, NSB and B0 (not TCT).
2. Pipette 100 μl standards into appropriate tubes.
3. Pipette 200 μl Tris-HCl buffer into NSB tubes and 100 μl Tris buffer into B0 tubes.
4. Pipette 400 μl Tris buffer into TCT tubes.

Samples and Reference Plasma (duplicates)

1. Pipette 200 μl sample or reference plasma into appropriate tubes.
2. Pipette 100 μl Tris buffer into sample and reference tubes.

Samples and Standards; General Assay Procedure

1. Add 100 μl 0.5% human globulin to all tubes.
2. Incubate for 15 min at room temperature.
3. Add 100 μl rabbit anti-PGFM J53 (1/15,0000) or J57 (1/8,000) or goat anti-PGFM 23 (1/6,000) to all

tubes except NSB and TCT. Anti-PGFM is stored at 1/100 in Tris-HCl.

4. Incubate for 30 min at room temperature.
5. Add 100 μl ^{3}H-PGFM (20 μL of stock (prepared 1-4-93) in 5 ml Tris) to all tubes (~18,000 dpm).
6. Incubate for 1h at room temperature and then overnight at 4^{0}C.
7. Add 750 μl PEG to all tubes and vortex for 1 min
8. Centrifuge for 30 min at 3,000 rpm at 4^{0}C.
9. Put tubes in foam racks, invert to drip dry (10 min).
10. Redissolve pellets in 750 μl Tris and vortex for 1 min.
11. Add 750 μl PEG to all tubes and vortex for 1 min.
12. Centrifuge for 30 min at 3,000 rpm at 4^{0}C.
13. Put tubes in foam racks, invert to drip dry (10 min.)
14. Redissolve pellets in 1 ml Tris and vortex for 3 min.
15. Transfer entire solution to scintillation vials.
16. Add 4 ml Bio HP to all vials and count.

Solutions

Buffer:

0.05 M Tris-HCl (7.88 gm/L Tris-HCl;)

0.1 % Na azide (1 gm/L;)

Adjust pH to 7.5 and store at 4°C.

Banamine-treated plasma:

Stored in 2 ml aliquots at -20°C.

Reference plasma:

High: 6.86 ml Banamine-treated plasma

140 µl 5,000 pg/100 µl Standard (50 ng/ml)

final concentration = 1 ng/ml

Low: 4.5 ml Bananime-treated plasma

0.5 ml High plasma (1 ng/ml)

final concentration = 100 pg/ml

Human Globulins:

0.5% w/v in 0.05 M Tris-HCl (fraction II, III from Sigma)

Polyethyleneglycol 8000 (PEG; Fisher Scientific, Inc.):

40% (w/v) in distilled water.

Standard Curve Stock Solution:

1 µg/ml PGFM in Tris-HCl buffer

Stored at -20°C

Standard Curve Working Solutions:

100 µl (1 µg/ml)+ 1.9 ml Tris buffer = 5,000 pg/0.1 ml

500 µl (5,000 pg/0.1 ml)+ 0.5 ml Tris = 2,500 pg/0.1 ml

400 µl (5,000 pg/0.1 ml)+ 1.6 ml Tris = 1,000 pg/0.1 ml

500 µl (1,000 pg/0.1 ml)+ 0.5 ml Tris = 500 pg/0.1 ml

500 µl (500 pg/0.1 ml)+ 0.5 ml Tris = 250 pg/0.1 ml

150 µl (1,000 pg/0.1 ml)+ 1.35 ml Tris = 100 pg/0.1 ml

500 µl (100 pg/0.1 ml)+ 0.5 ml Tris = 50 pg/0.1 ml

500 µl (50 pg/0.1 ml)+ 0.5 ml Tris = 25 pg/0.1 ml

100 µl (100 pg/0.1 ml)+ 0.9 ml Tris = 10 pg/0.1 ml

500 µl (10 pg/0.1 ml)+ 0.5 ml Tris = 5 pg/O.1 ml

APPENDIX B
PROTOCOL FOR OXYTOCIN RECEPTOR ASSAY
(PEG PROCEDURE)

TISSUE PREPARATION - wear gloves

1. Weigh 1 gm of tissue into weigh dish (keep frozen; return remaining tissue to -80°C immediately) mince with razor blade.
2. Transfer to 50 ml conical tube.
3. Rinse with 5 ml of homogenization buffer (HB; keep buffer on ice) and replace with 10 ml of HB.
4. Keep tube on ice until after the ultracentrifuge run, especially during homogenization.
5. Homogenize (5 sec on high; until smooth but not to the point that the mixture begins to turn grey or dark, grinding too long will denature the receptors). Transfer to ground glass homogenizer.
6. Homogenize 10 strokes with ground glass homogenizer. Pour into clean 50 ml tube. Rinse with 2 ml HB buffer.
7. Centrifuge at 3,000 X g for 10 min. Keep pellet for DNA assay (freeze in tube at -20°C).
8. Transfer supernate to ultracentrifuge tube. Balance exactly. Spin at 196,000 X g for 90 min.
9. Discard supernate.

10. Wash pellet twice with Membrane Diluting Buffer (MD), 1 ml.
11. Add 1 ml MD buffer, loosen pellet with transfer pipet, transfer to small ground glass homogenizer, homogenize until it is in solution.
12. Transfer to a polypropylene tube, rinse homogenizer with 1 ml MD buffer. Take out an aliquot for bicinchoninic acid assay. Equally divide the remainder into 2 separate tubes.
13. Freeze receptor tubes at -80°C and bicinchoninic acid assay tubes at -20°C.

Procedure for Oxytocin Receptor Assay

1. Label tubes (in triplicate) in order of; Total Count Tubes (TCT), Non-Specific Binding Tubes (NSB), Total Binding (Bo) (for Day 0 membrane preparation), Total Displacement (Bo+80) (for Day 0 membrane preparation), Total Binding (Bo) (for experimental tissue membrane preparation), Total Displacement (Bo+80) (for experimental tissue membrane preparation), Various levels of cold oxytocin for curve (experimental tissue).

   *(start numbering tubes at #2. The 1st tube will be a blank tube).

2. Make up radio-labeled oxytocin just prior to use (0.321 pM/100 μl).
3. Dilute Day 0 and experimental membranes (200 ug/100 μl) just prior to use.
4. Add 400 μl MD buffer to TCT, 200 μl MD buffer to NSB tubes, 100 μl MD buffer to Bo tubes.
5. Add 100 μl cold oxytocin at the appropriate level to the appropriate tubes (Bo+80 and curve).
6. Add 100 μl radio-labeled oxytocin (0.321 pM/100 μl) to all tubes.
7. Add 100 μl Day 0 membrane preparation. (200 μg/100 μl) to Day 0, Bo and Bo+80.
8. Add 100 μl experimental tissue membrane preparation. (200 μg/100 μl) to experimental tissue Bo, experimental tissue Bo+80, and curve tubes.
9. Incubate at room temperature (Make up gamma globulins (8 mg/ml) during incubation).
10. Set aside TCT (they will not have gamma globulins and PEG added or be centrifuged with the other tubes).
11. Add 100 μl gamma globulin (8 mg/ ml) to all tubes except TCT.
12. Mix by shaking rack by hand.
13. Add 1 ml 20% PEG (8000) to all tubes except TCT.
14. Vortex on speed 1.5 for 1 min.
15. Centrifuge at 3,000 X g for 10 min. (Label scintillation tubes while waiting).

16. Transfer spun tubes to foam racks. Drain supernatant into radioactive waste bucket. Drain tubes by inverting in foam racks over absorbent paper for 10 min.
17. Return tubes to tube racks. Add 400 µl 50 mM Tris vortex (speed 2) for 2 min.
18. Add 1 ml 20% PEG. Vortex (speed 2) 1 min.
19. Centrifuge and pour off as before.
20. Add 1 ml 50 mM Tris and vortex (speed 1.5) for 5 min.
21. Add 600 µl 50 mM Tris to TCT and move them back with the rest of the tubes.
22. Add 1 ml 50 mM Tris to the first scintillation tube to be used as a blank.
23. Pour off assay tubes into scintillation tubes. Add 400 ul 50 mM Tris to all assay tubes, vortex, and pour off into scintillation tubes again.
24. Add 4 ml Scintiverse II to scintillation tubes, cap, shake, and equilibrate 2 h
25. Count for 2 min.

Buffers For Oxytocin Receptor Assay

Homogenization buffer (HB)

50 mM Tris-HCl
250 mM Sucrose
4 mM EDTA

1. Adjust ph to 7.4 at 4°C (adjust pH close to 7.4, allow to equilibrate in cold room, and then adjust to final pH).
2. Keep sample on ice. If the temp. goes above 4° the pH will change considerably.

Membrane diluting buffer (MD)

50 mM Tris-HCl

1. Adjust pH to 7.4 at room temperature.
2. Use at room temperature. If used at cold room temperature the pH will harm the membrane preparations.

Oxytocin diluting buffer (OD)

50 mM Tris-HCl

0.2 % NaN_3

0.3 % BSA

20 mM $MnCl_2$

1. Add Tris, NaN_3, and BSA to 980 ml H_2O (if making 1L). Adjust pH to 7.5, equilibrate in cold room.
2. Add $MnCl_2$ to ~15 ml of water, once in solution equilibrate in cold room.
3. When both solutions are cold, slowly add $MnCl_2$ with a transfer pipet (if it falls out of solution it must be started again)
4. Check pH which must be at 7.4. If it is not then start over.

APPENDIX C
PROTOCOL FOR BICINCHONINIC ACID PROTEIN ASSAY

Assay Procedure

1. Use microtiter plates and run duplicates of each sample across the plate.
2. Pipet 100 µl SPB into wells A1 and A2.
3. Pipet 100 µl standard curves into wells A3-A12, B1-B4.
4. Pipet 100 µl known reference into wells B5 and B6.
5. Pipet 100 µl of unknown samples into wells B7-H12. (Dilute samples to between 5 and 10 µg/100 µl data points of the standard curve.)
6. Mix the working bicinchoninic acid (BCA) assay reagents just prior to use.
7. Pipet 100 µl BCA working solution into all wells.
8. Incubate 1 h at 60°C, cool to room temperature and read on microtiter plate reader Mode 89 at 540 nm wavelength or incubate 1 h at room temperature and read every 5 min for 1 h or until readings fall in center of curve.
9. Calculate mg protein/ml.

Solutions

Reagent A:

Na_2CO_3 6.84 w/v (Fisher Scientific, S-263-1)

NaOH 1.60 w/v (Fisher Scientific, S318B)

Sodium Tartrate Dihydrate 1.6 w/v (Sigma, S8640)

$NaHCO_3$ w/v (Sigma, S5761)

ddH_2O

Adjust pH to 11.25 with $NaHCo_3$.

Store at room temperature.

Reagent B:

Bicinchoninic Acid Disodium Salt 4.0% w/v

($C_{20}H_{10}N_2O_4Na_2$; Sigma S-8284)

ddH_2O

Store at room temperature

Reagent C:

Cupric Sulphate Pentahydrate 4.0% w/v

(Fisher Scientific, C-493)

ddH_2O

Store at room temperature

Sodium Phosphate Buffer (SPB) 0.1 M:

To prepare 1 liter of SPB (0.1 M): 77.4 ml Stock A +

22.6 ml Stock B + 900 ml ddH_2O

Stock A (1 M): 14.196 gm Na_2HPO_4 / 100 ml ddH_2O

(Fisher Scientific, BP 332-500)

Stock B (1 M): 11.996 gm $Na_2H_2Po_4$ / 100 ml ddH_2O

(Fisher Scientific, BP 329-500)

Store at room temperature or 4°C

Standard Curve:

BSA Stock Solution (50 μg/100 μl): 0.05 gm BSA /100 ml SPB

(Fraction V RIA grade; United Biochemical Corp., 10868)

1. 50 μl/100 ul
2. 25 μg/100 μl: 25 ml #1 + 45 ml SPB
3. 10 μg/100 ul: 10 ml #1 + 40 ml SPB
4. 5 μg/100 μl: 5 ml #1 + 45 ml SPB

APPENDIX D
PROTOCOL FOR OXYTOCIN RECEPTOR ASSAY
(FILTER PROCEDURE)

TISSUE PREPARATION - wear gloves

1. Weigh 1 gm of tissue into weigh dish (keep frozen; return remaining tissue to -80°C immediately) mince with razor blade.
2. Transfer to 50 ml conical tube.
3. Rinse with 5 ml of homogenization buffer (HB; keep buffer on ice) and replace with 10 ml of HB.
4. Keep tube on ice until after the ultracentrifuge run, especially during homogenization.
5. Homogenize (5 sec on high; until smooth but not to the point that the mixture begins to turn grey or dark, grinding too long will burn the receptors). Transfer to ground glass homogenizer.
6. Homogenize 10 strokes with ground glass homogenizer. Pour into clean 50 ml tube. Rinse with 2 ml HB buffer.
7. Centrifuge at 3,000 X g for 10 min. Keep pellet for DNA assay (freeze in tube at -20°C).
8. Transfer supernate to ultracentrifuge tube. Balance exactly. Spin at 196,000 X g for 90 min.
9. Discard supernatant.

10. Wash pellet twice with Membrane Diluting Buffer (MD), 1 ml.
11. Add 1 ml MD buffer, loosen pellet with transfer pipet, transfer to small ground glass homogenizer, homogenize until it is in solution.
12. Transfer to a polypropylene tube, rinse homogenizer with 1 ml MD buffer. Take out an aliquot for BCA assay. Equally divide the remainder into two separate tubes.
13. Freeze receptor tubes at -80°C and BCA tubes at -20°C.

Procedure for Oxytocin Receptor Assay

1. Label 18 12 x 75 mm borosilicate glass tubes (in duplicate) as Total Count Tubes (TCT) for each level of ^{3}H-oxytocin to be used (0.05, 0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 4.0, 8.0 pM/ 50 μl) and 36 tubes (4 tubes at each concentration of ^{3}H-oxytocin) for each experimental tissue sample.
2. Make up ^{3}H-oxytocin at concentrations listed above just prior to use.
3. Make up cold oxytocin (800 pM/ 50 μl)
4. Thaw experimental membranes preparations just prior to use.
5. Add 50 μl of each concentration of ^{3}H-oxytocin to appropriate tubes (2 tubes per concentration for TCT

and 4 tubes per concentration for experimental tissues).

6. Add 150 μl OD buffer to all TCT
6. Add 50 μl OD buffer to the first 2 tubes of 4 at each concentration of ^{3}H-oxytocin for experimental tissues to be used as an estimation of total binding.
7. Add 50 μl cold oxytocin to the last 2 tubes of 4 at each concentration of ^{3}H-oxytocin to be used as to estimate non-specific binding.
8. Add 100 μl experimental tissue membrane preparation all 4 of the tubes at each concentration of ^{3}H-oxytocin.
9. Incubate at room temperature for 60 min.
10. After incubation put on ice. Add 2 ml ice cold AW buffer to all tubes (TCT and experimental).
11. Transfer contents to Millipore GVWP 22μ membrane filters in filter manifold and apply vacuum.
12. Place filter in scintillation vial and add 4.5 ml Scintiverse II to scintillation tubes, cap, shake, and equilibrate 3 h or overnight.
13. Count for 5 min.
14 Calculate receptor concentration of receptors using the program Ligand or Scatchard analysis by hand.

DPM bound = DPM bound at each ^{3}H-oxytocin concentration - NSB at each ^{3}H-oxytocin concentration.

DPM total = total DPM at each concentration - NSB at each ^{3}H-oxytocin concentration.

DPM free = DPM total - DPM bound.

DPM are converted to mass and then to concentration using the specific activity of the radioligand and 2.22×10^{12} DPM/Ci. The regression of Y on X, where Y=[bound]/[free] and X=[bound], gives the receptor concentration (X intercept) and 1/slope (Ka) gives the Kd.

Buffers For Oxytocin Receptor Assay

Homogenization buffer (HB)

25 mM Tris-HCl

250 mM Sucrose

1 mM EDTA

1. Adjust pH to 7.4 at 4°C (adjust pH close to 7.4 then let equilibrate in cold room, before making final adjustment to pH 7.4).
2. Keep sample on ice. If the temperature goes above 4° the pH will change considerably.

Membrane diluting buffer (MD)

25 mM Tris-HCl

1. pH to 7.4 at 4°C.

Oxytocin diluting buffer (OD)

25 mM Tris-HCl

0.02 % NaN_3

0.2 % BSA

20 mM $MnCl_2$

1. Add Tris, NaN_3, and BSA to 980 ml H_2O (if making 1 L). Adjust pH to 7.5 at 4°C.
2. Add $MnCl_2$ to ~15 ml water and once in solution, equilibrate in cold room.
3. When both are cold slowly add $MnCl_2$ with a transfer pipet (if the $MnCl_2$ falls out of solution the procedure must be started again)
4. Check pH. It must be ~7.4, if not start over.

Assay Wash Buffer (AW)

25 mM Tris-HCl

0.02 % NaN_3

0.1 % BSA

10 mM $MnCl_2$

1. Prepare as OD buffer.

APPENDIX E
PROTOCOL FOR INOSITOL PHOSPHATE ASSAY

Preparation of Tissue

1. Each experimental tissue will be incubated in a pair of 20 ml glass vials. For each experimental tissue sample, thaw 2.5 ml KGI buffer and 2.5 ml BC buffer. Mix together and add 20 ml ddH_2O to make 25 ml KRB. Gas 5 min with 95% O_2 and 5% CO_2 by bubbling gas through buffer. Cap tightly and chill on ice.
2. After dissection of caruncular endometrium, immediately place approximately 0.5 gm tissue into 5 ml ice cold KRB in a petri dish on ice.
3. Cut tissue into pieces approximately 5-10 mg in size and place into petri dish with 5 ml (fresh) KRB on ice.
4. Remove tissue from buffer with forceps and rapidly blot on kim-wipe. Weigh 100 mg tissue directly into 20 ml borosilicate glass scintillation vials and place on ice.

Incubation

1. Add 10 μCi ^{3}H-inositol (50 μl) to each vial, swirl contents to break-up tissue clumps, gas as before, cap and return to ice.

2. Begin each incubation at 60 sec intervals by transferring vials to a Dubnoff metabolic shaking incubator at 37°C. Incubate 120 min to incorporate ^{3}H-inositol into cell membrane phospholipids.
3. After 120 min, remove KRB and discard. Replace with 1 ml fresh KRB previously warmed to 37°C, gas, cap and incubate for an additional 30 min.
4. After 30 min, remove KRB and discard. Replace with 1 ml fresh KRB warmed to 37°C. Add 20 μl 0.51 M LiCl (final concentration = 10 mM), gas, cap and incubate for 10 min.
5. Add 20 μl 0.1 M Na_2CO_3 to the first vial of each pair and 20 μl 5.2 μM oxytocin in 0.1 M Na_2CO_3 to the second vial of each pair (final concentration of oxytocin 100 nM), gas, cap and incubate 20 min.
6. Remove KRB and discard. Terminate incubation and lyse cells by adding 1 ml ice cold TCA. Place on ice for 30 min.
7. Transfer TCA to 15 x 85 mm borosilicate glass tubes. Rinse tissue with 200 μl ddH_2O.
8. Add 5 ml H_2O saturated diethyl ether to TCA tubes, cap with 15 mm polypropylene caps and extract with vigorous shaking for 5 sec. Carefully remove as much ether as possible with a pasteur pipette and discard. Repeat extraction procedure 4 times.

9. Move tubes to a 37°C H_2O bath. Dry off residual ether from aqueous phase under a stream of N_2 for 5-10 min. Neutralize with 25 μl 0.5 M Tris-HCl and store at -20°C until chromatographic separation of inositol phosphates.

Chromatographic Separation of Inositol Phosphates

1. Immediately prior to use, wash columns with three 5 ml volumes of ddH_2O.
2. Thaw samples and transfer to individual columns (12 samples can be eluted simultaneously). Wash each sample tube with 200 μl ddH_2O and transfer wash to its respective column.
3. Elute sample with three 3 ml volumes. Collect sample (contains inositol) and transfer 5 ml into 20 ml plastic scintillation vials.
4. Elute columns sequentially with 5 ml volumes of elution buffers #1, #2, #3, and #4, collecting each (containing glycerophospho-inositol, inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, respectively) directly into 20 ml plastic scintillation vials.
5. Add 15 ml scintillation cocktail (Scintiverse II) to each vial, shake, let equilibrate 2 h or overnight and count 5 min.
6. Values are expressed as DPM/gm wet tissue.

7. After use wash columns with two 5 ml volumes of elution buffer #5 and plug bottom. Add an additional 1 ml to top of column and cap. Columns can be stored as such and used for up to 10 elution procedures.

Buffers and Solutions

Kreb's Glucose Inositol (KGI):

9.0 gm Sodium Chloride ($NaCl_2$)

0.375 gm Potassium Chloride (KCl_2)

0.3 gm Calcium Chloride ($CaCl_2$)

0.035 gm Potassium Phosphate (KH_2PO_4)

0.03 gm Manganese Sulphate ($MgSO_4$)

1.80 gm Glucose

1.80 mg Myo-inositol

Bring up to 100 ml with ddH_2O. Store in 10 ml aliquots at -20°C

Bicarbonate (BC):

2.18 g Bicarbonate

Bring to 100 ml with ddH_2O. Store in 10 ml aliquots at -20°C

^{3}H-Inositol:

Bring 1.0 mCi to 5.0 ml in ddH_2O

Store at 4°C

Lithium Chloride (LiCl) 0.51 M:

0.51 M LiCl in ddH_2O

Store at 4°C

Sodium Bicarbonate (0.1 M $NaHCO_3$)

0.1 M $NaHCO_3$ in ddH_2O, adjust pH to 7.4

Aliquot and store at -20°C

Oxytocin (5.2 μM in 0.1 M $NaHCO_3$)

0.1 M $NaHCO_3$ (Prepared as above)

5.2 μM Oxytocin

Aliquot and store at -20°C

Trichloroacetic Acid

15% (v/v) in ddH_2O

Store at 4°C

Neutralization Buffer

0.5 M Tris-HCl, pH to 8.0

Store at 4°C

Elution Buffers

#1 - 25 mM Sodium Tetraborate

60 mM Formic Acid Sodium Salt

#2 - 0.1 M Formic Acid

0.2 M Formic Acid Sodium Salt

#3 - 0.1 M Formic Acid
0.4 M Formic Acid Sodium Salt

#4 - 0.1 M Formic Acid
1.0 M Formic Acid Sodium Salt

#5 - 0.1 M Formic Acid
2.0 M Formic Acid Sodium Salt

Store at room temperature

Column Preparation

1. Add Dowex-1 anion exchange resin (Sigma: 1 x 8 -200 Chloride Form) to an excess of ddH_2O.
2. Add slurry to Bio-Rad Poly-Prep columns with glass pipet to a volume of 0.6 ml.
3. Convert resin to formate form by sequential washing with 6 ml 1 N HCl, 6 ml 1 M NH_4OH and 6 ml elution buffer #5.
4. Store columns capped with enough elution buffer #5 to cover resin.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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